THE PHARMACOLOGICAL BEHAVIOUR

OF FELINE CORTICAL NEURONES

by

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ERRATA

Table of Contents -- in reference to p. 119, for "Purkinke" read "Purkinje."
List of Figures -- in reference to Fig. 23, for "DLH 'line' with" read "DLH 'in line' with"
-- in reference to Fig. 38, for "Purkinke" read "Purkinje."
Page 21, line 7 -- for "has" read "had"
Page 40, line 14 -- read "Doses of 10 μg. of N-methyl-D-aspartic acid, or 20-50 μg. of N-methyl-DL-aspartic acid (see Table II) produced....."
Page 43, Line 22 -- for "Section IV (c)" read "Section IV (d)"
Page 50, Line 21 -- for "porton" read "proton"
Page 53, Line 5 -- for "Drakonatides" read "Drakontides"
Page 59, Line 21 -- for "0.5 and 0.8" read "0.5 to 0.8"
Page 61, Line 19 -- for "to" read "do"
Page 62, Line 3 -- for "Electroncephalogram" read "electroencephalogram"
Page 64, Line 25 and Page 65, Line 1 -- for "Elliott, Khan and Jasper " read " Jasper, Khan and Elliott."
Page 72, Line 15 -- for "Fig. 13 A, B" read "Fig. 13"
Page 73, Line 10 -- for "40nA" read "50nA"
Figure 14, Legend -- for "40nA" read "50nA"
Page 78, Line 18 -- for "onset or excitation" read "onset of excitation"
Page 88, Line 13 -- for "as it was assumed" read "as was assumed"
Page 90, Line 16 -- for "62nA" read "65nA" and for "1.3" read "1.2"
Figure 18, Legend -- for "in the other cells" read "in other cells."
Page 92, Line 6 -- for "prolonger" read "prolonged"
Page 94, Line 12 -- for "100 V" read "100μV"
Page 102, Line 18 -- for "asymetry" read "asymmetry"
Line 21 -- for "of" read "on"
Page 103, Line 25 -- for "than the remaining" read "than ACh on the remaining"
Page 108, Line 6 -- for "othr" read "other"
Page 110, Line 9 -- for "sensitivity of ACh" read "sensitivity to ACh"
Figure 32, Abscissa -- for "6, 5, 4..0" of initial portion of time-scale, read "30, 25, 20...0"
Figure 32, Legend -- for "o.1" read "0.1"
Page 115, Line 2 -- for "antagonims" read "antagonism"
Page 123, Line 6 -- for "consited" read "consisted"
Page 129, Line 15 -- for "extraceullular" read "extracellular"
Figure 41A, Legend -- for "hydrachloride" read "hydrochloride"
Page 132, Line 21 -- for "d-dubocurarine" read "d-tubocurarine"
Figure 50, Legend -- for "cerveau izole" read "cerveau isole"
Page 150 -- delete lines 19 and 20
Page 155, Line 12 -- for "0.05-1ng/ml." read "0.05-1μg/ml."
Page 167, Line 8 -- for "comparisons" read "observations"
Page 169, Line 15 -- for "demonostration" read "demonstration"
Page 170, Line 22 -- for "Purkinke" read "Purkinje"
Page 173, Line 13 -- for "muscarninic" read "muscarinic"
Page 207, Line 6 -- read "Nature, 205, 603 - 604."

F. H. Crawford

Owing to the complex nature of the techniques involved, the majority of experiments involving microelectrophoretic ejection of drugs were conducted in collaboration with Dr D.R. Curtis.

However, the experiments of Sections III(e), IV(e) and Section X were entirely my own work except for the two experiments separately mentioned in 'Acknowledgments'.

J.M. Crawford

During the tenure of my scholarship at the Australian National University, the following papers detailing the results of these investigations have appeared or are in course of publication:-

Crawford, J.M. (1963) The effects upon mice of intraventricular injection of excitant and depressant amino acids (Biochem. Pharmacol., 12, 1443-44).

Crawford, J.M. and Curtis, D.R. (1964) The excitation and depression of mammalian cortical neurones by amino acids (Brit. J. Pharmacol., 23, 313-29).

Crawford, J.M. and Curtis, D.R. (1966a) Pharmacological studies on feline Betz cells - in course of publication.

Crawford, J.M. and Curtis, D.R. (1966b) Acetylcholine sensitivity of cerebellar neurones of the cat - in course of publication.

Crawford, J.M., Curtis, D.R., Voorhoeve, P.E. and Wilson, V.J. (1963a) Excitation of cerebellar neurones by acetylcholine (Nature, 200, 579-80).

Crawford, J.M., Curtis, D.R., Voorhoeve, P.E. and Wilson, V.J. (1963b) Strychnine and cortical inhibition (Nature, 200, 845-46).

Curtis, D.R. and Crawford, J.M. (1965) Acetylcholine sensitivity of cerebellar neurones (Nature, 206, 516-17).

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SECTION I - GENERAL INTRODUCTION

Although chemical synaptic transmission in the mammalian central nervous system and elsewhere is now widely accepted (see, for instance, Dale, 1952; Perry, 1956; Eccles, 1953, 1957, 1964; McLennan, 1963), any attempt to characterize the chemical transmitter substances involved must rest on several criteria which have been rigidly set forth by several workers, including Crossland (1957), Feldberg (1957), Paton (1958) and in the special case of inhibitory transmitters by Curtis (1961). From analogy with the steps by which acetylcholine was proved to mediate neuromuscular, vagal, and autonomic ganglionic transmission, these criteria include:-

(a) The natural occurence of the substance in the central nervous system, together with enzyme systems for its production.

(b) The storage of the substance at sites from which it is supposed to be liberated.

(c) Demonstration of the release of the substance from these sites during synaptic activation by physiological means.

(d) The identity of action on the postsynaptic cell of the synaptic process and the

substance presumed to mediate it. This criterion includes identity of behaviour towards pharmacological agents which facilitate, mimic or block these actions upon the postsynaptic cell membrane.

Certain problems must be overcome in the analysis of the action of possible transmitter substances on cortical neurones. These cells lie in the depths of tissues, invisible to direct observation, and form complex networks of synaptic interconnections. Microelectrode studies can however be made of their action potentials, and physiological techniques utilized in the identification of the cell type from which the spikes arise (e.g. Amassian, 1961). In this way, more detailed understanding of the synaptic organization of regions of simple cytoarchitecture is being reached (e.g. for the hippocampus, Andersen, Eccles and Løyning, 1963, 1964a, b; for the cerebellum, Andersen, Eccles and Voorhoeve, 1963, 1964; Eccles, Llinas and Sasaki, 1964, 1965a-g). Furthermore, by microelectrophoresis ionized drug molecules may be ejected into the close extracellular environment of single neurones in order to influence their behaviour. The advantages and limitations of this technique have been extensively reviewed by Curtis (1964).

In the present investigation, microelectrophoretic studies have been made of the behaviour of cortical neurones toward various amino acids related to L-glutamic and γ-aminobutyric acid, and toward acetylcholine and its congeners. The possible significance of these results for synaptic transmission in the cerebral, cerebellar and hippocampal cortices is discussed in the light of reported histochemical findings in Section XI.

In addition, some experiments on a possible common mode of actions of general anaesthetic agents on the cerebral cortex are reported in Section X.

SECTION II - EXPERIMENTAL METHODS

As virtually all experiments were performed upon cat preparations designed to expose either the pericruciate cortical gyri or the cerebellar vermis in the neighbourhood of the primary fissure, the standard preparative procedures will be outlined here, and any alternative techniques mentioned where appropriate in the following Sections.

(a) Exposure of the pericruciate cerebral cortex

Most experiments were performed on cats anaesthetised by intraperitoneal injection of sodium pentobarbitone (Nembutal, Abbott Laboratories; 35-40 mg./kg.), sodium diallylbarbiturate (Dial, Ciba Ltd.; 35-50 mg./kg.) or sodium diallylbarbiturate (100 mg./ml.) plus urethane (400 mg./ml.) (Dial Compound, Ciba Ltd.; 0.35-0.5 ml./kg.). In other cats the afferent pathways were destroyed by mid-collicular radio-frequency electrocoagulation (see (d) below).

The operative approach consisted of a unilateral removal of the bone overlying the anterior and posterior sigmoid gyri, the coronal gyrus, and the anterior lateral and anterior suprasylvian gyri. As a rule the exposure was left-sided, and extended 10-12 mm. laterally from

the mid-line, and 7-8 mm. caudal to the coronal suture. Anteriorly, it included the posterior wall of the frontal sinus. After incision and reflection of the dura, the exposed cortex was gently covered with small pieces of polyethylene or cellophane sheeting moistened with mammalian Ringer solution. A small area was left clear for subsequent insertion of the microelectrode, and the entire area was irrigated with warm (38^{o}C.) Ringer solution equilibrated with 95 per cent oxygen and 5 per cent CO_2.

The head was held in a modified Horsley-Clark headframe, and could be rotated in such a manner as to place the anterior or posterior sigmoid (pre- or post-cruciate) gyri uppermost and in an approximately horizontal plane.

It was possible to gently tease apart the pia-arachnoid mater from suitable areas with fine forceps, with minimal damage to the underlying cortex. This procedure greatly facilitated the insertion of the micro-electrode, and reduced the incidence and severity of pial dimpling. Cortical pulsations were minimised by the use of a small 'pressor plate' placed gently upon the surface without disturbing the circulation in the underlying cortical vessels (see Phillips, 1956), and the micro-electrode was inserted through a hole some 2 mm. diameter

in the centre of this plate. The area was kept under microscopic observation (x40 magnification, using a Zeiss 'Opton' binocular microscope) so that deterioration of the cortical circulation and 'clumping' of the erythrocytes in the vessels were apparent on their occurence. As a rule, the preparations remained in good condition for some twelve hours, but thereafter showed progressive deterioration in their cortical circulation and eventual depolarization of the cortical cells.

(b) Exposure of the cerebellar vermis

This was achieved by removal of an oval area of bone, some 8-10 mm. anteroposteriorly and extending 5-6 mm. on either side of the mid-line, just anterior to the lambdoid ridge. This area included the interparietal bone and portions of both parietals, and was usually sufficient to expose at least one folium of the vermis anterior to the primary fissure, and several caudal to it. Experiments were usually performed on lobules V-VI (Larsell, 1953; Jansen and Brodal, 1954).

The headframe was rotated about the axis parallel to the interaural line so as to place the desired folium uppermost, and the entire area was continuously flushed with warm carbogenated Ringer solution after the dura had been opened. Cortical stabilization was again achieved by the use of the 'pressor plate' (Phillips, 1956).

The pia mater was not removed from the cerebellar cortex, as it was impossible to do this without damaging superficial cells, but in young cats (weighing less than 3 kg.) the pia was usually sufficiently tenuous to permit insertion of a 4-6 micron microelectrode without undue dimpling. As a rule, cells could be recorded at depths of as little as 60μ beneath the surface in these preparations.

(c) Exposure of the hippocampal cortex

The left hippocampus was approached through a wide craniotomy in the temporo-parietal region, incision and reflection of the dura, and removal of the cortex and white matter of the marginal, suprasylvian and ectosylvian gyri by suction. The exposed dorsal aspect of the hippocampus and hippocampal fimbria were continuously irrigated with the warm carbogenated mammalian Ringer solution used in cortical experiments elsewhere. The 'pressor foot' was used to stabilize the hippocampal cortex also for microelectrode recording.

(d) Cerveau isolé preparations

After induction using halothane (Fluothane, I.C.I. Ltd.) on a face mask, a glass T-tube was inserted and tied into the trachea, and anaesthesia continued using 2 litres/

minute of 2-3 per cent halothane vapour mixtures on 'open circuit'.

Three alternative modes of preparation were tried on various animals, either supra- or immediately subtentorial approaches using a blunt knife to sever the brainstem, or a subtentorial approach for an electrocoagulation technique using radio-frequency current between needles inserted stereotaxically.

(i) The supratentorial approach, with bilateral craniotomy and wide incision of the dura over the occipital poles, suffered from major disadvantages in that the cerebral hemispheres were disturbed during the transection, and because of difficulty of avoiding damage to the great cerebral vein.

(ii) The subtentorial approach was made through the anterior portion of the cerebellum. When transection was made by a blunt knife or spoon, visualization of the inferior colliculi and brainstem was aided by partial ablation of the anterior portion of the cerebellum by suction, but the electrocoagulation procedure could be carried out without this step.

The rack of electrodes used for radio-frequency electrocoagulation consisted of four stainless steel needles held parallel to each other in a transverse plane, each 2 mm. apart and insulated with Araldite coating resin

except for 1-1½ mm. bare area at their tips. The assembly was carried on a micromanipulator (Narishige, Tokyo, Japan) and could be inserted stereotaxically at an angle of 30° to Horsley-Clark vertical, just posterior to the tentorium. The manipulator was previously calibrated to establish the position H.C.C. 0-2 mm. anterior, -10 mm. vertical, and coagulation was performed by passing current (500,000 cycles per second; 30-40 mA) for 15-20 seconds between adjacent pairs of needles. After coagulation at each level, the rack was withdrawn in steps of 1½-2 mm., and the process repeated a total of six or seven times.

The halothane anaesthetic was then discontinued, and clinical decerebrate rigidity supervened within 2-5 minutes. The pupils, which had been dilated during the coagulation, became constricted (in a few cases asymmetrically), and failed to react to painful stimuli to the paws, body or face. Reflexes were active, in some cats to such a degree that the animals required to be paralysed and artificially ventilated. Respiration and femoral arterial bloodpressure were unaffected by the coagulation.

Post-mortem section of the brains of these preparations revealed gross coagulation of structures for some 4 mm. on either side of the mid-line, in an oblique plane 1-2 mm. thick passing dorsoposteriorly from the pyramidal tracts, and involving the pontine nuclei, medial lemniscus

and part of the pontine reticular formation, medial longitudinal bundle and brachium conjunctivum.

(e) Pons and medullary pyramids

A ventral approach was used, with section of the trachea and oesophagus below the level of the larynx and gentle dissection of the cranial stump rostrally until the basi-occiput and auditory bullae were exposed. The longus cervicis muscles were then freed from the basi-occiput to clear the inferior margin of the foramen magnum, and the bone removed and dura incised to expose the lower portion of the pons, the ponto-medullary junction, and the pyramids on the ventral aspect of the medulla.

Stimulation of the pyramidal tracts was by just-suprathreshold square wave pulses (0.2 msec. duration) from bipolar electrodes placed on the surface of the ipsilateral pyramids rostral to their decussation.

The pontine nuclei were stimulated through a concentric needle electrode, insulated except at its tip, and inserted stereotaxically to a point H.C.C. 1-2 mm. anterior, -8 to -10 mm. vertical, and 1-2 mm. lateral to mid-line (Snider and Niemer, 1961). The nucleus tegmenti pontis was found by advancing the stimulating point to about -5 mm. vertical.

(f) Cerebellar nuclei (ipsilateral fastigial, external cuneate and lateral reticular nuclei, and contralateral inferior olivary nuclei) were stimulated electrically by means of concentric steel needle electrodes insulated with Araldite coating resin except at the tip, where both the inner wire and the outer barrel (a No.23 hypodermic needle) were exposed and ground back to a plane inclined face. The electrodes were each carried on separate small manipulators attached to the headframe, and the nuclei were sought stereotaxically after removal of the posterior portion of the occipital bone between the lambdoidal ridge and the foramen magnum, extending laterally in an irregular oval for some 6-8 mm. from the mid-line.

The co-ordinates used were based on the atlases of Reinoso-Suárez (1961) and Snider and Niemer (1961), and articles by Brodal (1943) and Grant (1962) on the lateral reticular and external cuneate nuclei. At the conclusion of each experiment, iron was deposited at the site of stimulation by passing direct currents of 3-5 mA for 15-20 seconds from the outer barrel of each electrode as anode to the indifferent electrode in the scalp muscles of the cat. These deposits were then 'developed' by immersion of the cerebellum and brainstem in 10 per cent formol saline containing potassium ferrocyanide, and the

position of the dense blue colouration checked 2-3 days later macroscopically and histologically.

The usual Horsley-Clark stereotaxic co-ordinates employed were:-

(i) Fastigial nucleus: $8\frac{1}{2}$-10 mm. posterior, 0 to -1 mm. vertical and $1\frac{1}{2}$-2 mm. ipsilateral.

(ii) Inferior olivary complex: 9-10 mm. posterior, -10 to -11 mm. vertical and 1-2 mm. contralateral.

(iii) External cuneate nucleus: Electrode placed just on or immediately beneath the dorsal surface of the medulla, at level of obex (14 mm. posterior) and 4-$4\frac{1}{2}$ mm. ipsilateral. It was thus just rostrolateral to the surface markings of the cuneate nucleus itself, and was readily re-positioned in order to obtain a suitable evoked potential at the cerebellar vermis.

(iv) Lateral reticular nucleus: 10-11 mm. posterior, -9 to -11 mm. vertical and 3-4 mm. ipsilateral.

(v) Pontine nuclei, including nuc. tegmenti pontis, were approached from the ventral aspect (see preceding section). Either ipsilateral or contralateral nuclei could be stimulated, as their projection to the cerebellar vermis

consists of both crossed and uncrossed fibres (Jansen and Brodal, 1954, chapter II).

(vi) In a few experiments, the superficial and deep radial nerves of the right forearm were placed on stimulating electrodes in a pool of warm paraffin oil, and the responses of cells of the ipsilateral vermis studied.

(g) General preparative procedures for experiments

When the dissections had been completed, the animals were suspended by a thoracic and a pelvic clamp, with the abdomen and thorax held clear of the base of the animal frame. The temperature of the preparations was kept constantly at 36-38°C. by means of two heating elements, one of which was controlled by a thermistor probe placed beneath one scapula. All animals had an intravenous cannula inserted into the cephalic vein of one forearm for the administration of intravenous agents, including gallamine triethiodide where paralysis and artificial ventilation were required. Many other cats, including all cerveau isolé preparations, had in addition a femoral arterial cannula from which the mean arterial blood pressure could be intermittently or continuously monitored by means of a Statham transducer (type P23Db).

In almost all experiments, a five-barrel micropipette was used both for extracellular ejection of drugs and the simultaneous recording of cell spike activity. Some of the experiments in the cerebellar cortex required two micropipettes on separate micromanipulators (see Section II(i), and Fig. 4), and in these cases either one five-barrel assembly for ejection and local recording and a 'distant' single-barrel recording pipette, or two multibarrel micropipettes were used. In the latter case, mutual interaction could be studied.

The centre barrel of all multibarrel pipettes contained 4-5 M NaCl solution for extracellular recording, while the outer barrels contained aqueous solutions of the drugs under test.

Freshly-pulled five-barrel pipettes were broken back with a fine glass rod under microscopic control until their overall tip diameters were 4-8μ. Each individual orifice would then be 0.7-1.5μ (assuming the same ratio of internal to external diameter of the tubing at the tip as before pulling). This size represents a suitable compromise between the low rate of drug ejection possible with smaller orifices of high electrical resistance, and the probability of tissue damage by larger microelectrode tips.

Solutions of the drugs to be tested were prepared in glass-distilled water, and the pH adjusted as necessary (using a glass electrode measuring system) to a value suitable for electrophoretic ejection either as a cation or an anion (see Curtis and Watkins, 1963). After removal of any suspended particulate matter by centrifugation, each solution was introduced into a barrel of the micropipette with fine polyethylene tubing, and filling was completed by further centrifugation (3,000-3,500 r.p.m. for 7-10 minutes) (see also Curtis and Davis, 1963).

The filled micropipettes were inspected under a water-immersion objective before use to ensure that all barrels were completely filled and that the orifices were at the same level. This ensured that drug ejections were made near the point of recording, and minimized the possibility that irregularity of the microelectrode tip could cause unnecessary tissue damage. Electrodes were again inspected after each series of tracks to ensure that they had not become broken during use, and to determine the presence and amount of 'capping' of the electrode tip by myelin and cellular debris which may have interfered with the ejection of drugs from the micropipette barrels (see Andersen and Curtis, 1964a).

Whenever storage of filled micropipettes was necessary they were stored in a refrigerator (3-4°C.), with the tips immersed in water or 0.9 M NaCl solution. Storage was either in air or under nitrogen in a large closed vessel. Almost invariably the filled micropipettes were used within three days of preparation.

The centre (NaCl) barrel of the assembly was connected by means of a small silver-silver chloride junction to a negative-capacitance cathode follower and thence to the amplifying and display system, as described by Andersen and Curtis (1964a). This gave a direct record of the firing frequency of the selected cell, as well as filmed records of all the electrical activity recorded by the microelectrode (see also Fig.1). Where extracellular records were obtained simultaneously from separate cells using two microelectrodes (Section II(i)), two such 'firing-frequency indicating systems' were used, each with separate ratemeter and display facilities. The firing-rate of each cell was separately plotted on two channels of an Offner Dynograph paper recorder, while a third channel was utilized to show the passage of the currents which ejected drugs from each pipette.

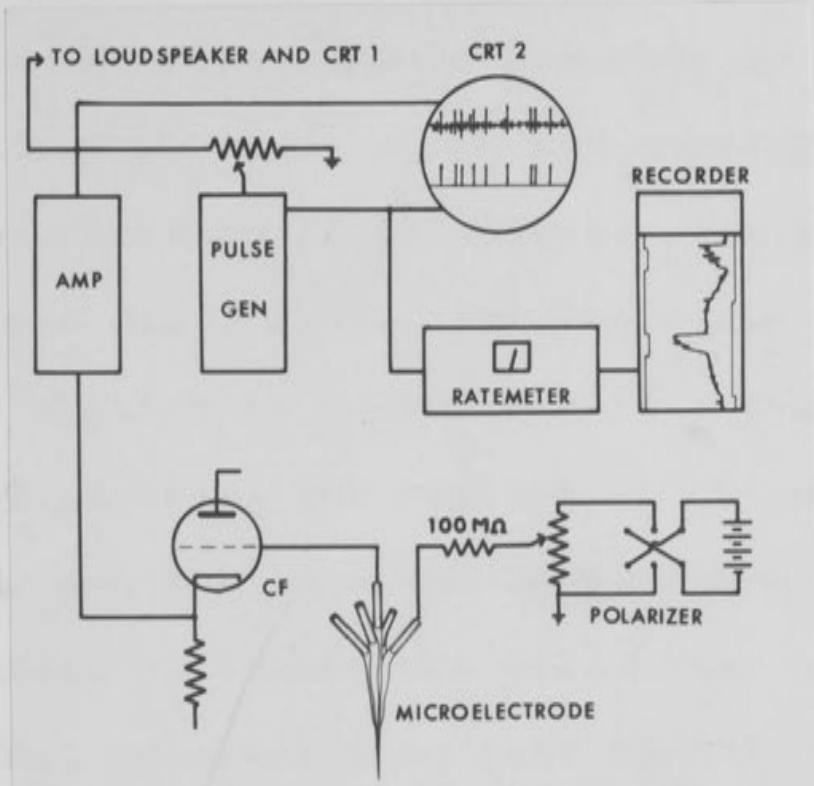

Fig. 1: Schematic diagram of the experimental arrangement.

The polarizers are used to retain drugs in, or eject them from, the outer barrels of the microelectrode. Electrical activity recorded by the centre barrel of the microelectrode passes a neutralized capacitance cathode-follower pre-amplifier (CF) and after amplification is displayed on two oscilloscope screens (CRT 1 and 2). Portion of the amplifier output is used to trigger a pulse generator whose output pulses are counted by a ratemeter. Simultaneously, these pulses are displayed with the triggering spikes on the second beam of CRT 2. A direct plot of the firing-frequency is made concurrently by the pen recorder (Recti-Riter, Texas Instruments Inc.).

(h) Criteria for identification of single neurones

(i) Betz cells. These neurones were identified by constant latency antidromic response to stimulation of the medullary pyramid, which was exposed from the ventral aspect (see Section II(e) above). The latency range accepted was 0.5-5 msec., and the cells were also tested for their ability to follow repetitive stimuli (Phillips, 1956, 1959, 1961). Two volleys to the pyramidal tract were used, and the interval between them reduced until the second failed to invade the cell under observation. If this minimal interval were less than 1 msec. (e.g. Fig. 2), the identification of the cell as a Betz cell was considered proven, but values less than 1.5 msec. separation were taken to indicate a cell which could be classed as 'possibly Betz' (see Table V). Relatively few cells, however, were found to fall within this class.

All other cortical neurones were grouped together as non-Betz cells, although it is realized that the stimulus strength applied to the pyramids in a particular case may not have reached the threshold for the axon of the cell under observation, and such a cell would therefore fail the first test. Alternatively, the stimulus strength used may have brought in a number of extraneous inhibitory circuits whose convergence upon the cell being tested prevented it from following two antidromic volleys at

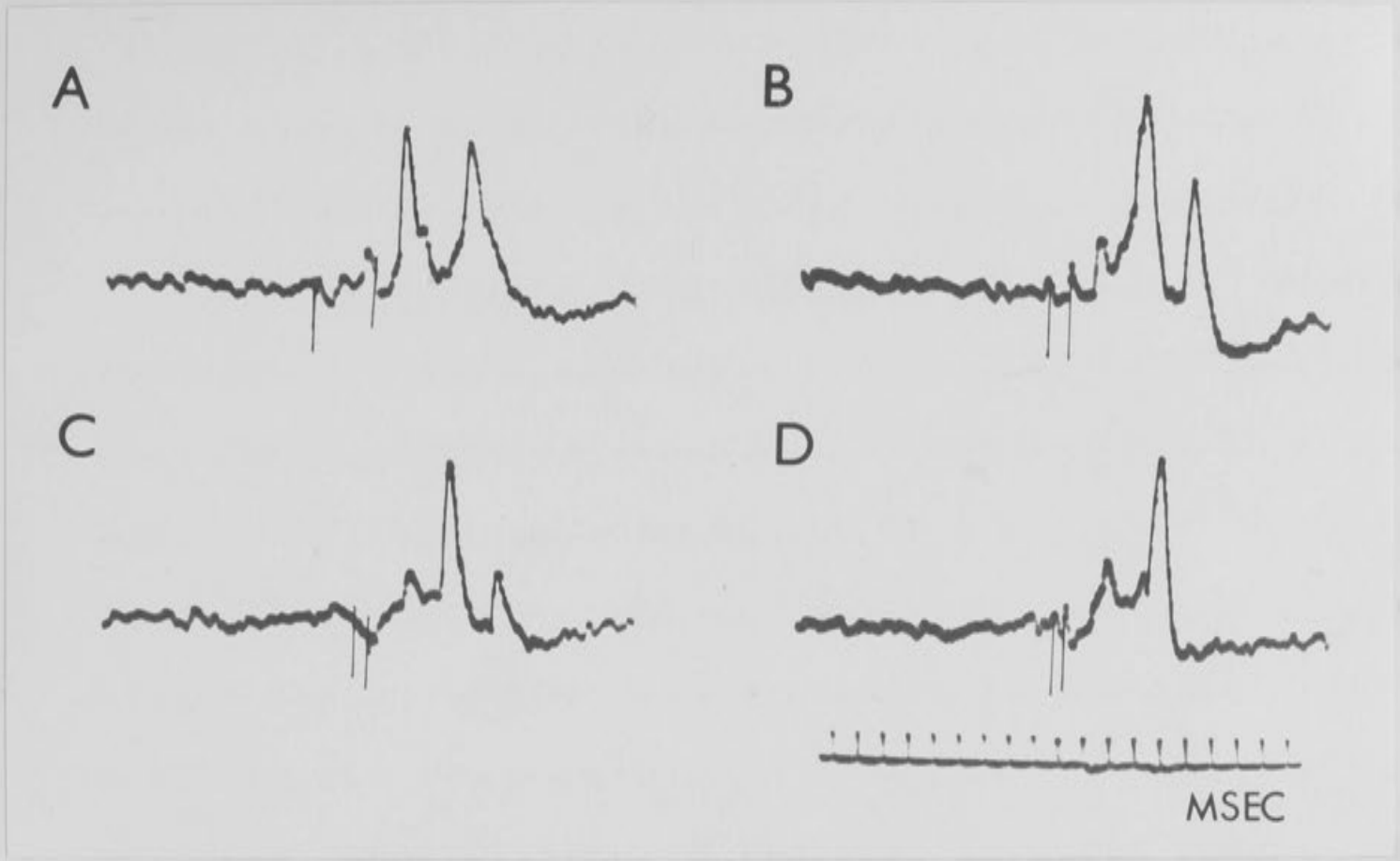

Fig. 2: Identification of a Betz cell. Two just-supra-threshold volleys to the medullary pyramids were delivered at decreasing intervals (A, 2.35 msec; B, 0.82 msec; C, 0.58 msec; D, 0.42 msec) in order to determine the critical interval within which the second impulse failed to invade the cell. The latency of antidromic invasion of this cell was 3.46 msec.
Time scale: msec.

short intervals (see Phillips, 1959). Thus, although all the cells classified here as Betz cells undoubtedly were of this type, many pyramidal tract neurones will be included in the 'insufficiently identified' categories.

(ii) Cerebellar Purkinje cells (P-cells). Because of the usual perpendicular approach to the folial surface, the most superficial P-cell layer was reached through a minimum of cortical tissue, and at a depth of 0.2-0.4 mm. In addition to the depth of the recording, two or more of the following criteria were used to identify those cells which were reported as P-cells:-

(1) Short-latency antidromic invasion from stimulation of the ipsilateral fastigial nucleus (Granit and Phillips, 1956) (Fig. 3B).

(2) Rather longer latency orthodromic firing by strong stimuli to the fastigial nucleus or its close vicinity (juxta-fastigial or JF stimulation), which presumably spread to excite nearby climbing and/or mossy fibres (Granit and Phillips, 1956; Eccles, Llinas and Sasaki, 1964a).

(3) Orthodromic activation, with 1.5-4 msec. latency, by 'inline' bipolar surface stimulation of the parallel fibres of the same folium (Dow, 1949; Andersen, Eccles and Voorhoeve, 1963). This excitation was frequently followed by a positive potential, averaging

10-15 msec. in duration (Fig. 3C), which was attributed to the effect of basket and stellate cells also fired by the parallel fibre system (Eccles, Llinas and Sasaki, 1965g).

(4) Prolonged depression of spontaneous or amino acid-induced firing by 'out of line' bipolar surface stimulation. This is due to an activation by the parallel fibre system of basket and stellate cells which impinge on the observed P-cell, and hyperpolarize it (Andersen, Eccles and Voorhoeve, 1963, 1964). For any given P-cell, the duration of this 'pause' in the firing was approximately inversely proportional to the rate of firing beforehand (Fig. 3D and 41).

(5) Orthodromic activation and repetitive firing by electrical stimulation of the climbing fibres originating in the inferior olivary nucleus (Dow, 1939; Jansen, 1957; Szentagothai and Rajkovits, 1959; Eccles, Llinas and Sasaki, 1964).

(6) The occurence of spontaneous 'inactivation responses' with repetitive firing and changes in spike shape (Granit and Phillips, 1956). These appear to be the result of activity in the climbing fibre system (Eccles, Llinas and Sasaki, 1964) (Fig. 3A).

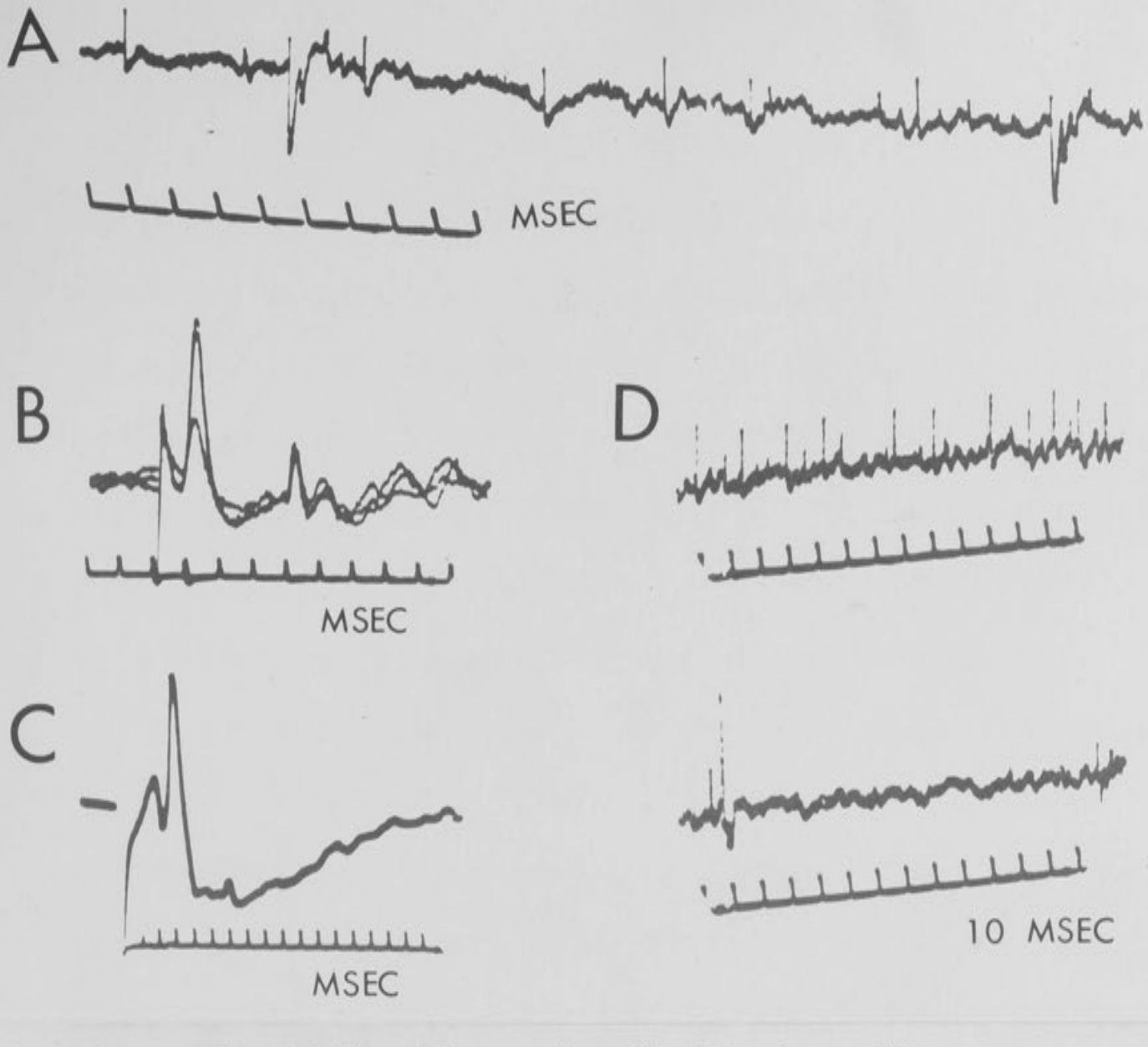

Fig. 3: Identification of a Purkinje cell.

A: Spontaneous firing of a cell, showing "inactivation complexes".

B: Antidromic invasion (0.83 msec latency) from juxta-fastigial stimulation.

C: Effect of local bipolar surface stimulation of parallel fibre system. An initial negativity is followed by a prolonged positive potential (see text).

D: Effect of "out of line" parallel fibre stimulation on the amino acid-induced excitation of a Purkinje cell. In the lower trace, surface stimulation resulted in 130 msec cessation of firing (for further description see text).

Time Scales: msec (A, B, C); 10 msec (D).

It was not possible to use all of these criteria for each cell, particularly as the area of tissue containing neurones which could be fired antidromically by electrical stimulation of the fastigial nucleus by the stereotaxically-placed bipolar electrode was usually very small. Owing to the relatively large size of the concentric bipolar electrodes it was felt that any attempt to reposition the stimulating electrode in this region would cause undue damage to the nucleus. In general, Purkinje cells were identified by the comparative ease with which negative-positive (200μV to 1 mV) extracellular spike potentials could be recorded at depths of 0.2-0.4 mm., by the spontaneous firing which could be depressed for periods of up to 500 msec. by local surface stimulation of the folium, and by the presence of spontaneous 'inactivation complexes'.

(iii) Granular-layer cells of the cerebellum. With the relatively large micropipettes used (4-8μ overall tip diameter), it was virtually impossible to record extracellular spike responses of single cells located at depths exceeding some 0.4 mm. Attempts to identify granule cells failed, whether by antidromic activation via surface stimulation of the parallel fibres or by orthodromic stimulation from mossy fibres, and it appears that the microelectrode tip must have damaged many of the

neighbouring granule cells during its insertion. As a rule at these depths numerous small spikes representing activity in very many neurones were recorded simultaneously, but it was impossible to select any one neurone from such groups in order to count its discharge frequency.

(i) 'Two electrode' experiments in the cerebellum

After a Purkinje cell has been located with one microelectrode, either a single NaCl-filled pipette or the central barrel of a five-barrelled microelectrode, its activity was recorded extracellularly as a second microelectrode was used to eject drugs upon (and record the resulting activity of) cells in synaptic relationship with the first.

This technique was devised in order to take advantage of the highly ordered and laminar arrangement of the cerebellar cortex, and to determine whether granule or basket cells were cholinoceptive by observation of their effects on P-cells. Such an indirect approach is necessary because of the difficulty of physiological identification of neurones other than P-cells by extracellular recording techniques alone (see also Eccles, Llinas and Sasaki, 1965g). The microelectrode arrangement used is shown diagramatically in Fig. 4.

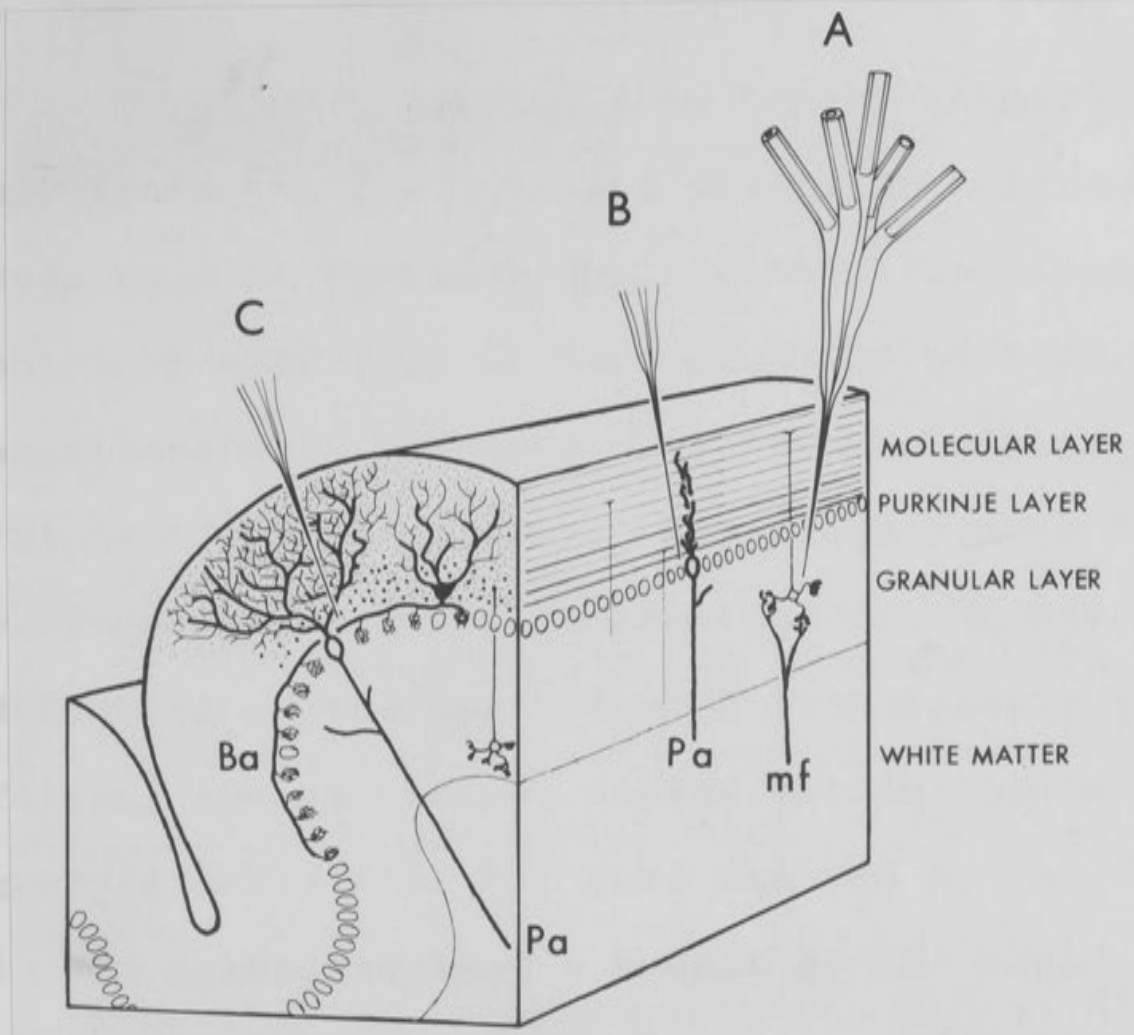

Fig. 4: Schematic arrangement of "two-electrode" experiments in a cerebellar folium (shown cut in longitudinal and transverse planes). Drug ejections were made by electrode "A", and the resultant local activity recorded. At the same time, the activity of an identified Purkinje cell was also recorded by another microelectrode at "B" (in the longitudinal axis of the folium) or at "C" (transversely separated from the plane through "A"). Further description in text.

Drugs were ejected from one five-barrelled microelectrode ('A') at various points in a folium while the responses of Purkinje cells either 'in-line' with the point of ejection, or 'out of line' with it, were simultaneously recorded with another electrode at position 'B' or 'C' respectively. Experiments were made with various longitudinal separations of the electrodes, differing separation measured transversely across the folium, and at various depths within the cortex. If granule cells near 'A' were excited by the drug ejection, it was predicted that a P-cell at 'B' would also be excited by the ensuing activity in the parallel fibres (granule-cell axons). On the other hand, a P-cell such as that recorded by electrode 'C', transversely separated by 100-500μ from the folial axis through 'A', would not be fired by these parallel fibres, but show postsynaptic inhibition as a result of basket and stellate cell activity (see Andersen, Eccles and Voorhoeve, 1963, 1964; Eccles, Llinas and Sasaki, 1965d,g). One such basket cell, with its axon (Ba) spreading transversely to the P-cells near 'C', is shown in Fig. 4. Finally, when ejections were made with the second electrode transversely across the folium from the first, it might be practicable to excite basket cells near one electrode which end upon

a P-cell recorded with the other. In order to reduce the number of attempts necessary to obtain a successful interaction of this type, both electrodes were multi-barrelled to enable ejection and recording to be performed by each alternately.

(j) Other experimental series

Experiments were also carried out upon spinal interneurones in the lumbar region of cats anaesthetized with pentobarbitone sodium, the preparation being as described by Curtis, Phillis and Watkins (1959).

The actions of systemically administered convulsants (strychnine, picrotoxin and pentamethylenetetrazol) upon the recurrent inhibition of Betz cells and the 'pause' in Purkinje-cell firing which followed local surface stimulation of the folium are described in Section IX (a) and (e).

A few experiments involving intracerebral and intra-ventricular injections of amino acids were performed on mice (Section III(e) and IV(e) below) using the technique of Haley and McCormick (1957), and some estimations of the effect of anaesthetics on muscarinic acetylcholine-responses were performed on guinea-pig ileum in vitro (Section X(f)).

SECTION III - ACIDIC AMINO ACIDS

(a) Introduction

Investigations in the mammalian central nervous system to date have failed to reveal any serious discrepancies in the proposals that electrophoretic ejection of certain acidic amino acids causes excitation of the neurones close to the ejecting pipette, whereas the corresponding members of the neutral amino acid series cause neuronal depression (see Curtis and Watkins, 1960a,b; 1963, 1965).

Experiments have now been reported with these compounds on neurones in the cat spinal cord (Curtis, Phillis and Watkins, 1960; Curtis and Watkins, 1960, 1963), brainstem (Curtis and Koizumi, 1961; Bradley and Wolstencroft, 1962, 1965), thalamus (Curtis and Andersen, 1962; Andersen and Curtis, 1964a) and lateral geniculate nucleus (Curtis and Davis, 1962), cerebellum (Krnjević and Phillis, 1963a; Crawford, Curtis, Voorhoeve and Wilson, 1963), cerebral cortex (Krnjević and Phillis, 1963a; Crawford and Curtis, 1964; Krnjević, 1964, 1965a), hippocampal cortex (Biscoe and Straughan, 1965; Stefanis, 1964) and retina (Noell, 1960), and electrophoretic ejection of L-glutamic or DL-homocysteic acid is becoming

widely used as a means of exciting quiescent neurones for pharmacological study (e.g. McCance and Phillis, 1964a, b; Krnjević, Randić and Straughan, 1964). The remarkable uniformity of the pattern of excitation or depression produced by any one amino acid ejected near neurones in various portions of the central nervous system has led to alternative hypotheses:- an action, unrelated to synaptic function, at 'two-point' or 'three-point' receptors upon the nerve cell membrane (Curtis and Watkins, 1960); or else an almost universal central transmitter function for members of the amino acid series (see, for example, 'Inhibition in the Nervous System and GABA', ed. E. Roberts; and also Krnjević, 1964, 1965a). The most likely candidates as transmitters are L-glutamate and γ-aminobutyric acid (GABA), which are known to exist in relatively large amounts in nervous tissue, and have recently been shown to be released from the cerebral cortex in amounts which vary with the state of cortical activity (Jasper, Khan and Elliott, 1965). Yet again, it is possible that some of these substances have a non-synaptic regulatory function in the nervous system. Curtis and Watkins (1965) point out a close correlation between the reported rates of active uptake of certain amino acids by brain slices from suspension media, and the potency of the same compounds upon

mammalian central neurones. To date, only a few compounds closely related to L-glutamate and to γ-amino-butyric acid have been examined in this manner, and it would be of interest to determine whether the correlation also holds for the more potent synthetic members of each amino acid series. If this be so, these authors suggest that the pharmacological action of the amino acids may be attributed to ionic movements associated with their uptake by the cells rather than to any synaptic activation.

The present experiments, which involve extracellular recording of the action potentials of various neurones in the cerebral, hippocampal and cerebellar cortices, were not designed to elucidate details of the mode of action of amino acids on these cells, but to compare the parameters of excitation and depression produced by the amino acids with those already reported from other sites in the nervous system.

For convenience, the results obtained with acidic (excitant) amino acids are presented in this Section, and those with neutral amino acids, amines and other related compounds, in Section IV(a-e). The discussion of both groups of results follows Section IV.

(b) Acidic amino acids upon cerebral cortical neurones

Pericruciate neurones were studied in both the pre- and post-cruciate gyri of 14 anaesthetised and 9 cerveau isolé cats. The majority of those neurones which could be identified as Betz cells by antidromic invasion from the medullary pyramids (Phillips, 1956, 1959, 1961) were firing spontaneously at 5-20/second, usually in a random fashion or in the form of 'spindles' (Dempsey and Morison, 1943), and were found at depths between 0.8 and 1.3 mm. of the surface (cf. Krnjević and Phillis, 1963a). Betz cells, however, did not differ from other pericruciate neurones in their sensitivity and responses to acidic amino acids. In two preliminary experiments also, neurones in the visual area (both striate and parastriate cortex; cf. Doty, 1958) were investigated, again with comparable results. All cortical neurones were accordingly grouped together, and this term is used in this and the following Section to denote a cell within 2 mm. of the cortical surface from which spike responses could be produced by ejection of an excitant amino acid.

The acidic amino acids were ejected as anions from solutions of pH 8.0-8.6, measured by a glass electrode system before the micropipettes were filled, and checked after the experiment by withdrawing a small amount of the solution.

It was usually possible to move the micropipette until the size of extracellular spike of the major unit under observation was 250 to 500 μV (negative-positive), and could readily be distinguished from the smaller spikes of more distant cells. Under such circumstances the effects of current flow associated with electrophoretic ejection were minimal (see Curtis and Koizumi, 1961), and were distinguished from actions due to the amino acid by testing the effect of current flow through the micropipette containing sodium chloride. In some instances the spike responses of a particular neurone could be identified over a distance of 50-100μ, but there was no appreciable change in the relative potencies of the amino acids at different levels of spike size.

Cortical neurones were found to undergo 'depolarization block' at firing frequencies of about 20-60/second when excited by the acidic amino acids (Fig. 5). This phenomenon, in which the initially negative-positive spike potentials were converted either to predominantly positive spikes, or merely decreased in size before failing entirely, has been attributed to the change in membrane conductance and the depolarization produced by the excitant (Curtis, Phillis and Watkins, 1960). A further feature frequently observed under these conditions is a widening of the spike potential of the cell. Cells of

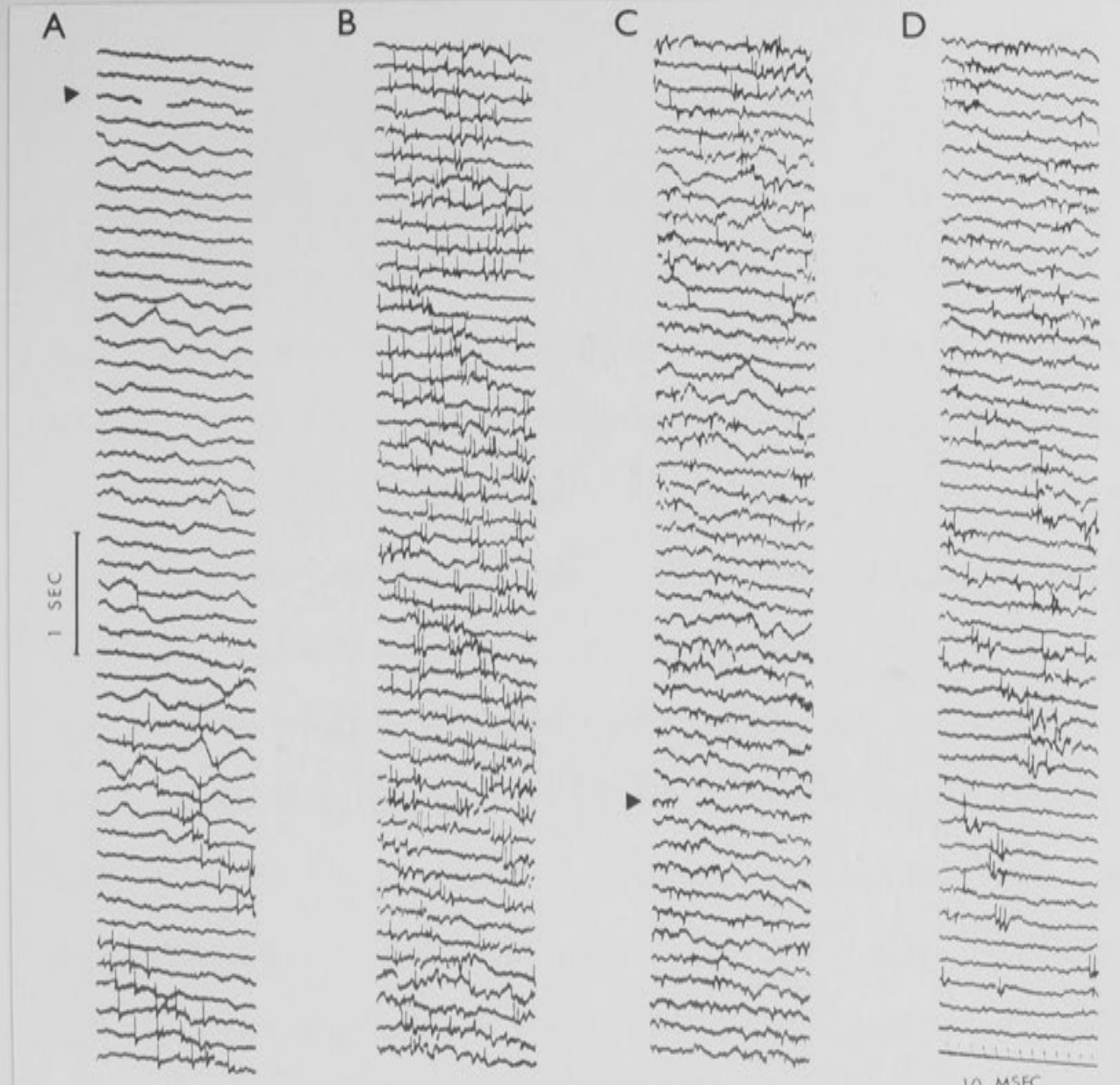

Fig. 5: The development of a depolarization block. Continuous filmed records (reading from above downwards, and from left to right) were made. At the first signal (near the top of column "A"), N-methyl-D-aspartic acid was ejected with a current of 23 nA for 23 sec, its termination being marked by the signal in column "C". A progressive increase in firing rate, followed by spike changes, is evident. After the end of the amino acid ejection, spikes transiently reappeared (column "D").
Time scale: 10 msec.
Rate of film movement shown by 1 sec. time-scale next to column "A".

the cerebral (and cerebellar) cortex tend to undergo these alterations in spike shape with excitation by DLH or L-glutamate at lower firing frequencies than have been observed with spinal interneurones, thalamic and geniculate neurones, although it is possible to fire cortical neurones synaptically (or antidromically in the case of Betz cells) at much higher rates than some 60 spikes/second. Depolarization block has not yet been seen with excitation by cholinomimetics, although the latter substances were capable of firing cortical neurones as rapidly as did the amino acids. The greater readiness of DLH to produce depolarization block when compared with ACh or carbaminocholine may merely reflect a more extensive area affected by the conductance change, or may involve differences in the ionic species participating in the depolarization by amino acids and by cholinomimetics.

It is possible that the acidic amino acids produce 'depolarization block' so readily because of a pre-existing depolarization due to tissue hypoxia. Disturbances in cerebral cortical circulation must necessarily be associated with the surgical procedures despite care in preparation, a normal level of systemic blood pressure, and the constant irrigation of exposed cortical tissue. The alteration produced in the pressure relationships within the cranial cavity by opening the

skull, particularly when the ventricular system was partially collapsed as a result of opening the dura in the region of the pyramids, inevitably leads to circulatory disturbances and changes in cerebral blood flow. Corpuscular clumping was usually obvious in the smaller cortical veins; such a phenomenon is rarely seen in spinal cord preparations where the exposed tissue is covered with a pool of warmed paraffin oil. Deterioration of the circulation and heightened sensitivity to acidic amino acids were most evident towards theend of experiments which lasted 12 to 18 hours.

It is impossible to assess the drug concentration produced at a cell by local electrophoretic ejection, but by assuming that the distance between barrels of a multibarrel micropipette is small compared with the distance between any barrel and the cell, and that equal currents through the various barrels eject the contained substance from each at more or less equivalent rates, relative potencies may be calculated. The difficulties associated with these assessments have been fully discussed by Curtis and Watkins (1963), and do not require further comment here.

Where records are obtained on many occasions, with consistent values of the relative potencies toward a reference compound such as L-glutamic acid, some degree

of confidence exists as to the validity of the results. In the present series, the ratio of ejecting currents which produced equal firing frequencies (i.e., the ratio of concentrations for equal effects) were compared, the duration of the ejections being sufficiently long for a stable firing-rate of the cortical neurone to be established for each amino acid. Where possible, comparisons of potency were repeated also at different firing rates on the same cell. Compounds were tested upon four or five cells in each of two or three preparations, using fresh micropipettes and varying combinations of the amino acids on each occasion. The range of potencies and the mean value (relative to L-glutamate) for the acidic amino acids tested are given in Table I, together with an estimate of the latencies of onset and offset of excitation, and the number of separate tests.

These potency-ratios, as well as the time-parameters of excitation of cortical neurones by each of the acidic amino acids, are very close to those reported for spinal neurones (Curtis and Watkins, 1960b and 1963). Also, as has been found in other parts of the central nervous system, the excitant actions of the acidic amino acids upon cortical neurones were readily reversible and recovery occurred even after long periods of depolarization block produced by excessive amounts of these compounds. Following

excitation, particularly at high frequencies, by the amino acids it was usual to observe a period during which the spontaneous firing of the cell was depressed or absent. This depression lasted as much as 30 seconds in some cases, and was again a feature common to both cortical and spinal neurones.

(i) N-methyl-D-aspartic acid (NMDA) was the most potent excitant amino acid of those tested, and had a prolonged duration of action after the electrophoretic current was terminated (see Curtis and Watkins, 1963). In some cases, diffusion of this substance from a 0.1 M solution was sufficient to fire neurones. When ejected with currents of 3-10 nA NMDA produced a gradually increasing rate of firing over 15-30 seconds (see Fig.6), and the rate declined over a period of 2-10 seconds after the ejection had ceased. When larger amounts of NMDA were used (currents of 12-30 nA) the cell increased its rate of firing more rapidly until a depolarization block ensued, the spike then being indistinguishable from the background noise. Simultaneously the spontaneous and antidromically-evoked spikes of the neurone were also blocked. Several seconds after the current which ejected the amino acid was terminated, spikes would often reappear transiently in a burst of 1-2 seconds duration, presumably as portions of membrane, which had been rendered incapable

of generating spikes by the amino acid, recovered while the level of depolarization remained above the threshold for spike generation.

It has been reported (Krnjević and Phillis, 1963a) that N-methyl-D- and N-methyl-DL-aspartic acid caused a prolonged reduction in the sensitivity of cortical neurones to L-glutamic acid. Many tests of this interaction were carried out, but the finding could be repeated only if sufficient NMDA had been administered to produce a block of neuronal spikes. The results from one such experiment are shown in Fig. 6. Initially three successive ejections of L-glutamic acid (28 nA) gave reasonably consistent firing rates of about 20/second. Following this, the ejection of NMDA (7 nA) produced a firing frequency of 15-20/second, with no change in the shape of the spike potential (compare A and B), and the firing produced by the following dose of L-glutamate (within 3 seconds of the end of the ejection of NMDA) was unaffected. However, when a current of 12 nA was used to eject NMDA a maximum frequency of approximately 30 spikes per second was attained, the spike was altered in shape, and the cellular responses were eventually blocked (C). Subsequently, two doses of L-glutamic acid produced only small positive spikes (D) but 35 seconds after the cessation of the NMDA ejection, L-glutamic acid again

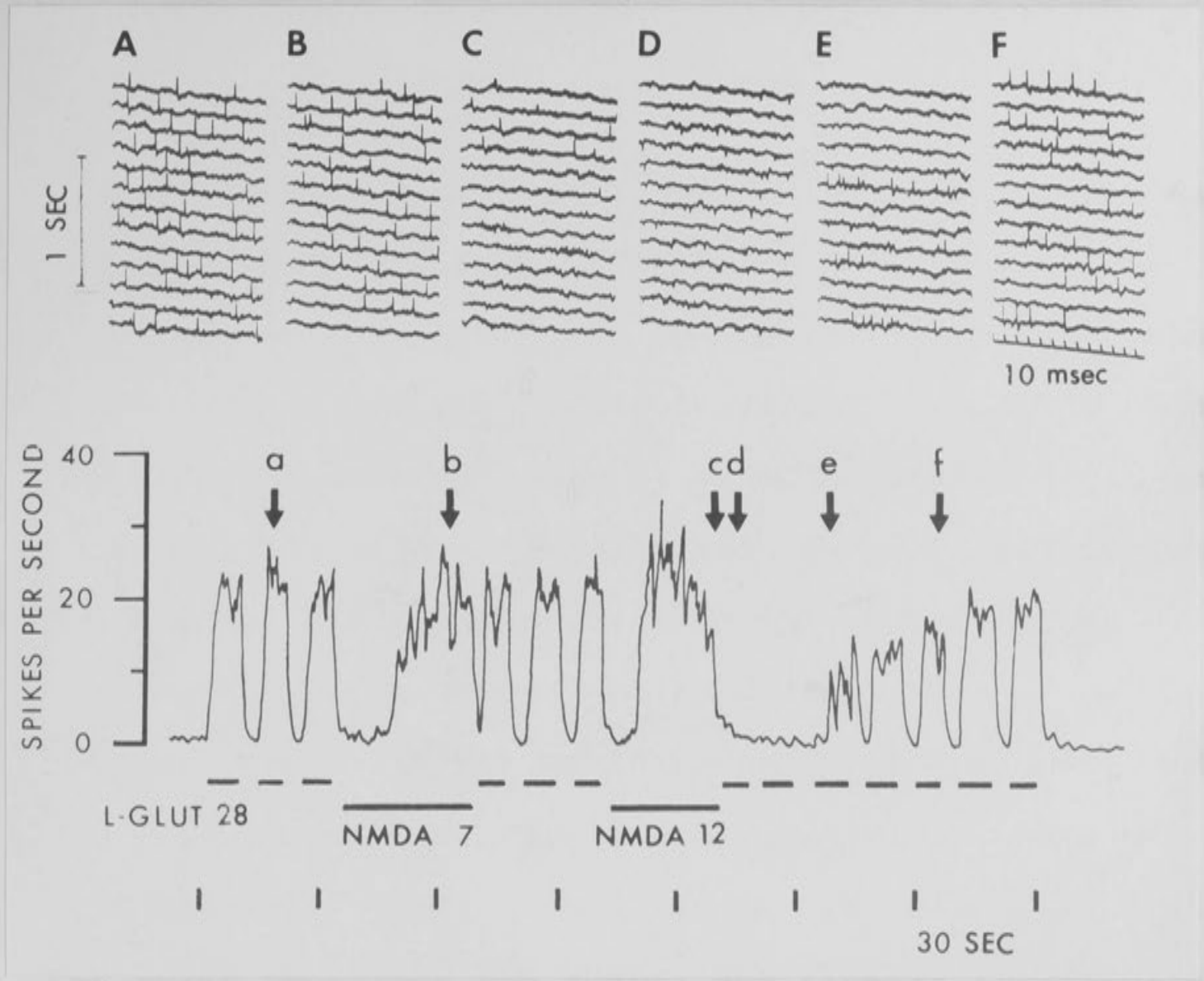

Fig. 6: The interaction of N-methyl-D-aspartic acid (NMDA, lower bars) and L-glutamic acid (upper horizontal bars) on Betz cell. The continuous trace shows the firing frequency of the cell, and portions of the concurrent filmed record (A-F) are reproduced above this trace to show the spike shape at the corresponding points (a-f) of the firing-frequency record.
Ordinate: Firing frequency in spikes per second.
Abscissa: Time in 30 sec. periods for paper recorder; 10 msec. for filmed records, with rate of film movement given by 1 second scale by column A.

fired the cell with negative-positive spikes. Initially these spikes were small (compare E with A) and full recovery of spike size (F) was observed after one minute.

Although in most cases the onset of complete block by NMDA was comparatively slow, with several cells and particularly with electrophoretic currents greater than 20 nA, the onset was abrupt and the spikes suddenly vanished with little or no preliminary alteration in shape. Following the administration of sufficient NMDA to supress cellular responses the usual sequence of recovery was return of antidromic invasion, restoration of spike amplitude and shape, and finally re-establishment of the sensitivity to L-glutamic acid.

Similar observations have been made upon the apparent reduction in sensitivity of neurones to L-glutamic acid by prior administration of DL-homocysteic acid (Fig. 7) and N-n-propyl-D-aspartic acid, and also to test doses of DL-homocysteic acid by N-methyl-D-aspartic and L-glutamic acids. Furthermore, experiments upon spinal interneurones have failed to demonstrate any depression of the firing induced by either L-glutamic or DL-homocysteic acid by a preceding dose of N-methyl-D-aspartic acid. In fact, as occasionally observed with cortical cells, the effectiveness of L-glutamic or DL-homocysteic acid was actually increased when these were ejected shortly after

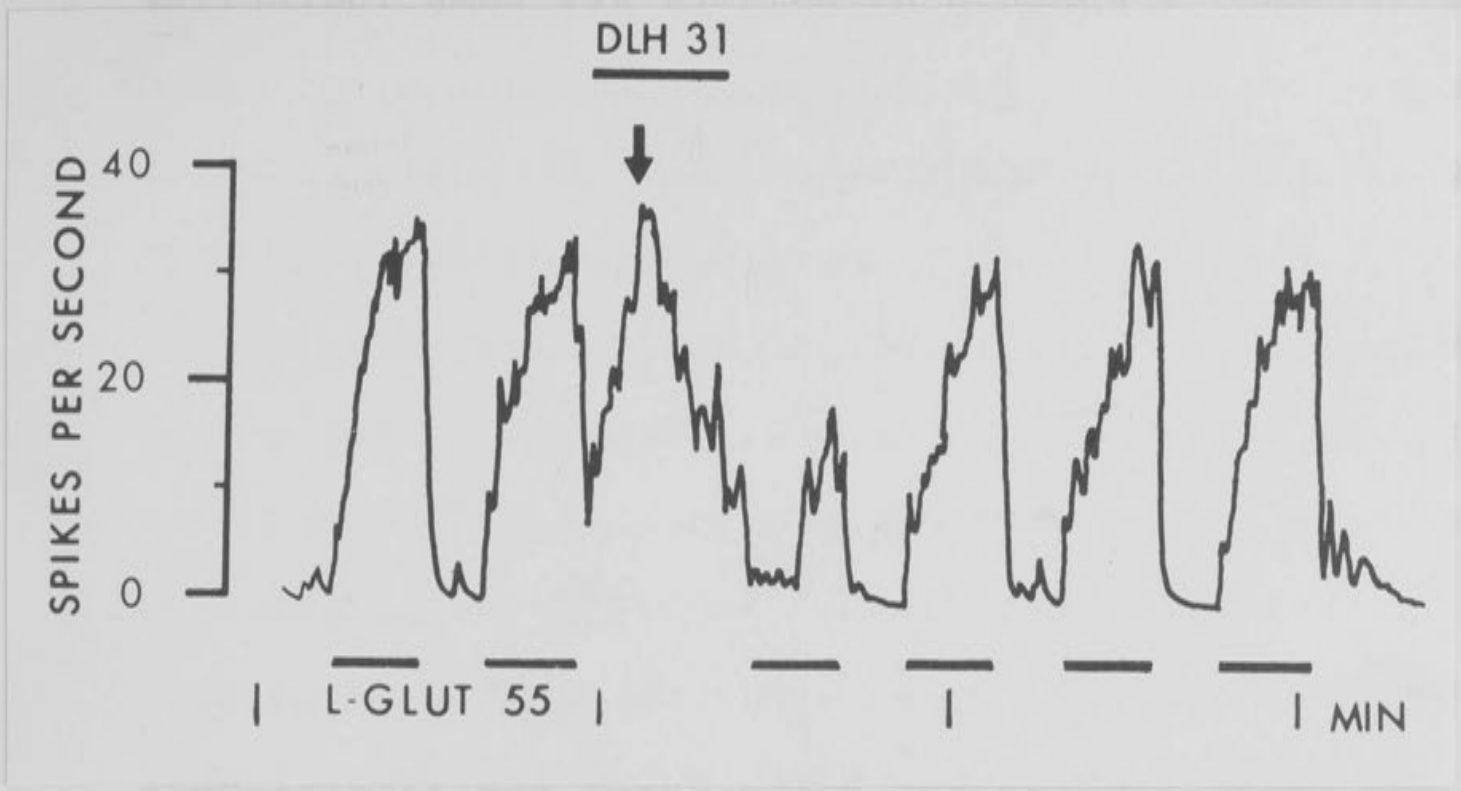

Fig. 7: The interaction of DL-homocysteic acid (DLH) and L-glutamic acid. The trace shows the firing frequency of a Betz cell in response to ejections of L-glutamic acid (L-glut, 55 nA) before and after sufficient DLH had been ejected to produce a depolarization block (current of 31 nA). Increasing positivity of the spike occurred at the point signalled by the arrow, and thereafter the response to L-glutamate ejection was reduced for $2\frac{1}{2}$ minutes. Ordinate: Frequency in spikes per second. Abscissa: Time in minutes.

the administration of NMDA was terminated. When sufficient NMDA was ejected to produce a 'depolarization block' of spinal interneurones the effects of subsequent doses of other excitant amino acids were reduced, just as with cortical neurones.

Several cortical cells were tested with graded doses of N-methyl-D-aspartic acid and it was found that there need be a difference of only 10-20 per cent between currents (concentrations) which produced neither 'depolarization block' nor a reduction in L-glutamate sensitivity, and those which did produce block and a reduction in the sensitivity. It was thus concluded that the diminution in amino acid sensitivity was primarily a consequence of alterations in membrane conductance and potential, rather than of receptor desensitization due to a prolonged occupation of amino acid receptors by the N-alkyl excitant amino acid. This phenomenon is possibly related to the frequently observed reduction in the spontaneous discharge rate which follows high frequency amino acid induced firing of spinal, thalamic and cortical neurones.

(ii) N-n-propyl-D-aspartic acid (NPDA) has been tested on only three Betz and three unidentified cortical neurones. On each, however, its potency was comparable to or slightly greater than that of L-glutamate. The

onset of firing with this substance was gradual, the rate increasing progressively over as much as 15 seconds. The offset after the end of administration of NPDA was as a rule 10-20 seconds, but facilitatory effects on brief test doses of L-glutamate could be demonstrated for up to 1 minute after the end of NPDA ejection, using small electrophoretic currents. As had been observed with NMDA, however, larger amounts of N-n-propyl-D-aspartic acid also caused a depolarization block and subsequent depression of the sensitivity to L-glutamate.

(iii) DL-homocysteic acid (DLH) had a potency $2\frac{1}{2}$-6 times that of L-glutamic acid, and showed a relatively slow onset and offset of excitation (Curtis and Watkins, 1963). While the time taken to reach maximal firing frequency differed somewhat from cell to cell, presumably because the distance from the pipette orifice to the receptive areas of the cell varied, it was never as long as with NMDA, being usually some 10-20 seconds. Similarly the offset period was shorter (2-5 seconds).

(iv) Glutamic and aspartic acid isomers. The potency difference between these compounds is shown in Table I, and although high accuracy cannot be claimed for the estimations, particularly as only a limited number of neurones were tested, the observed differences are consistent with those found on spinal neurones (Curtis and

Watkins, 1963) and in the thalamus (Andersen and Curtis, 1964a). As explained above, the comparison was based upon the currents required to produce equal rates of firing. The potencies therefore reflect theratios of concentrations necessary for equal effects, a method generally accepted when comparing the activity of pharmacological agents interacting with a common receptor. This method is preferable to a comparison between effects produced by equal extraneuronal concentrations (see Krnjević and Phillis, 1963a, page 282), since differences in response and therefore potency ratios by this latter method will depend upon the slope of the respective portions of the dose-response curves under study.

Both D- and L-glutamate and D- and L-aspartic acids had rapid onset and offset of excitation. The maximal firing rate was reached within 0.5 to 1 second, and at the end of the electrophoretic current flow firing ceased within a similar time. As with spinal interneurones, the D- and L- forms of these amino acids could not be distinguished on time factors alone.

(c) Acidic amino acid actions upon cerebellar neurones

In most experiments, intermittent ejection of DL-homocysteic acid as the micropipette was lowered through the cerebellar cortex enabled the presence of otherwise

quiescent cells to be readily established. There appears to be no difference in susceptibility between Purkinje cells, unidentified cells of the molecular layer (presumably including basket and stellate cells), and the groups of cells encountered in the granular layer towards this amino acid. In a few comparisons, DLH was approximately three times as potent as L-glutamate, but no systemic survey of relative potencies has been attempted. In general, excitation of cerebellar neurones by acidic amino acids follows the same time course as does that of cortical (and spinal) neurones.

(d) Acidic amino acid actions upon hippocampal cortex

DL-homocysteic acid was ejected upon all 36 cells investigated, at depths of 0.2 to 1.4 mm. beneath the hippocampal surface. The cells were uniformly excited, with a latency of some 2-5 seconds for the onset and offset of amino acid action, and readily showed the spike changes characteristic of 'depolarization block'.

When ejected at depths of 0.29-0.79 mm., DLH produced characteristic synchronized positive-negative waves at a frequency of 25-40 spikes per second, usually preceded by spike activity in neurones close to the micropipette. These waves were of maximal amplitude at about 0.3-0.5 mm., and were found to reverse in polarity as the microelectrode

was lowered further (Fig. 8). Similar waves have also been described by Biscoe and Straughan (1965). The frequency of these amino acid-evoked waves is much higher than that of the hippocampal 'theta rhythm' (Green and Arduini, 1954; Green, Maxwell, Schindler and Stumpf, 1959; Spencer and Kandel, 1962), but the underlying mechanism of synchronization of the firing of pyramidal cells is probably similar. It appears that the amino acid depolarizes the pyramidal cells, which discharge impulses along their axons and axon collaterals. Basket cells are thereby excited, and produce widespread post-synaptic inhibition both of the cells originally excited and all other pyramidal cells upon which their terminals impinge. The duration of basket-cell inhibitions in the hippocampus is normally more than 100 msec. (Andersen, Eccles and Løyning, 1963, 1964a, b), but will be curtailed in the present case by the depolarization of the pyramidal cells due to the continued presence of the acidic amino acid.

The hippocampal waves first appeared some 5-10 seconds after the start of DLH ejection, and outlasted the amino acid administration by a similar period. These latencies are somewhat longer than for the excitation of single cells by the amino acid, and presumably indicate

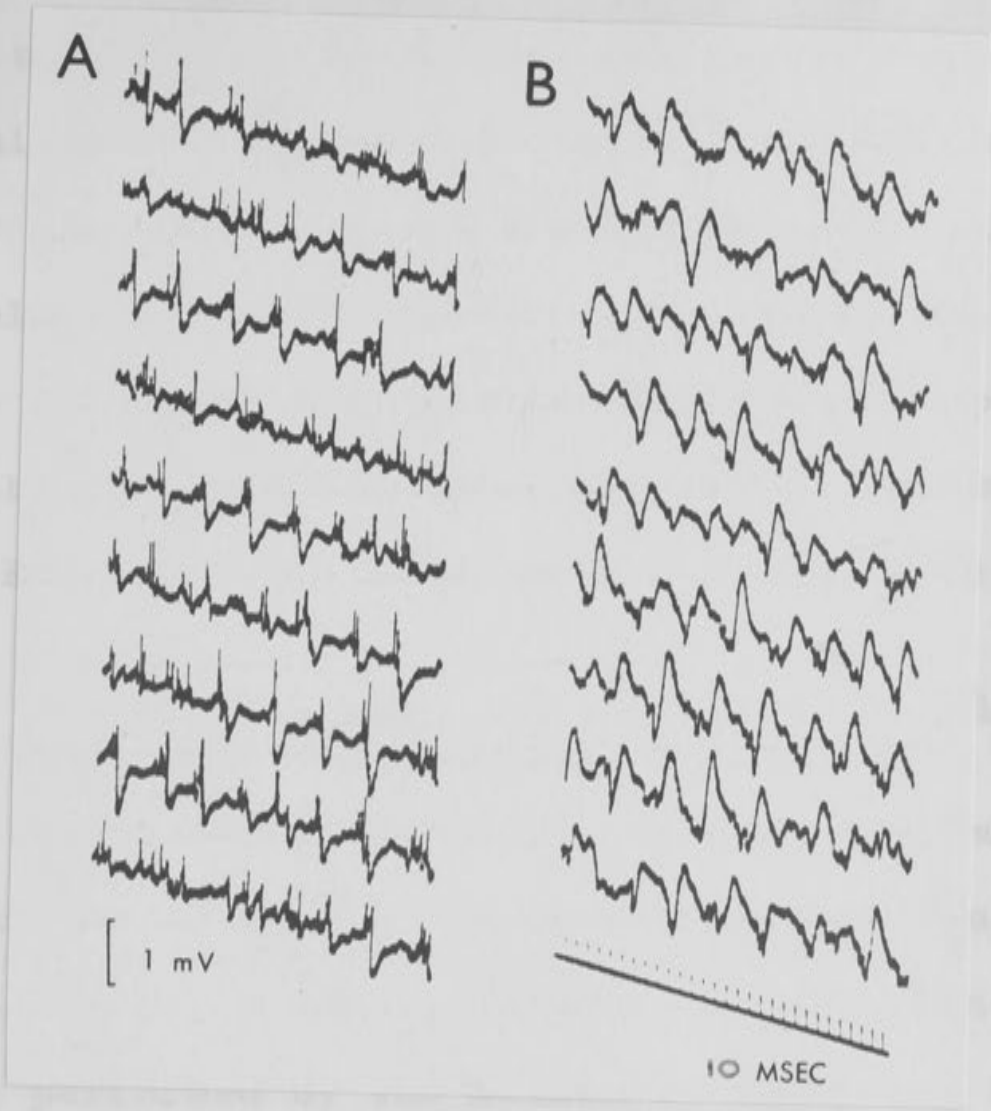

Fig. 8: Filmed records of activity elicited in the hippocampal cortex by ejection of DL-homocysteic acid.

A: Microelectrode tip at 0.29 mm depth, the record commencing 10 seconds after the start of DLH ejection with a current of $6\frac{1}{2}$ nA.

B: Microelectrode tip at 0.5 mm depth, 10 seconds after the start of DLH ejection by $6\frac{1}{2}$ nA.

Both records read from above downwards.

Time scale: 10 msec (both records).

the time taken for diffusion to affect a considerable population of pyramidal cells around the microelectrode tip, and thereby start the synchronized alternation of depolarization and basket-cell inhibition.

As essentially similar mechanism of phasing by inhibitory feedback operates in the thalamus (Andersen and Eccles, 1962; Andersen and Sears, 1964).

(e) Intraventricular and intracerebral injection of excitant amino acids in mice

Intraventricular injections of 0.05 ml. volumes of solutions of various isomers of N-methyl-aspartic, homocysteic, N-methylglutamic, aspartic and glutamic acids were performed by the method of Haley and McCormick (1957) into four-week-old mice.

Doses of 10 μg of N-methyl-DL-aspartic acid produced clonic flexor convulsions, followed by extensor spasm and death. Smaller doses of these excitants, or doses of 20-50 μg of D- or DL-homocysteic acid produced a pattern of gross hyperactivity, with running, leaping and violent scratching at the head or body. Flexor convulsions were frequently observed as part of this response, and the period of hyperactivity was followed by one in which the animals were subdued and their responses to disturbances were sluggish. This phase of recovery lasted as much as

half an hour, after which the animals appeared normal. Still smaller doses of these more powerful excitants, or injection of 50 µg of N-methyl-L-aspartic, N-methyl-DL-glutamic, L-homocysteic, D- or L-aspartic, or 150 µg of L-glutamic acid, caused mild hyperactivity.

A full resumé of the results of these experiments will be found in Table II. In view of the large number and variety of neurones affected by such relatively drastic treatment, the close parallel between the order of relative potency in these experiments and those involving electrophoretic ejection near single neurones is remarkable. The earlier findings of Kita, Kamiya and Kiyota (1963) and of Gulati and Stanton (1960) are also in essential agreement as to the relative potency of various acidic amino acids using this rather crude method of estimation.

SECTION IV - NEUTRAL AMINO ACIDS AND RELATED COMPOUNDS

(a) Introduction

Neutral amino acids related to γ-amino-butyric acid (GABA) reduce the sensitivity of neurones in the cortex to depolarization by acidic amino acids, and to excitation by other means, including the spontaneous synaptic firing of the cells. The potency of these depressants can be most readily assessed in terms of the electrophoretic current needed to produce a specified reduction in the firing rate of a cell excited by an acidic amino acid. In the present series, DL-homocysteic acid was used as the control excitant substance, and was ejected either continuously or at fixed intervals and for constant times (see Fig. 9A-C).

Table III lists the neutral amino acids and other compounds used in this study, together with the concentrations and pH of the solutions. Potencies are expressed relative to that of GABA (---), the number of symbols indicating greater (----), less (--) or very weak (-) activity.

In the absence of intracellular records from Betz cells it was necessary to try and establish the possible mode of action of GABA and related compounds on the basis

of extracellularly recorded potentials. Grundfest and his collaborators (see Grundfest 1961, 1964; Purpura 1959, 1960) have proposed that GABA specifically blocks the superficial excitatory axodendritic synapses of cortical neurones. In the present experiments the action of GABA has not been determined upon spikes elicited by different synaptic pathways, and it could be argued that the depression of spontaneous activity observed is consistent with Grundfest's proposal. Furthermore, it might also be suggested that the depression by GABA of the firing produced by DL-homocysteic acid was associated with competition between these amino acids at receptor sites beneath axodendritic excitatory synapses.

However, if the action of GABA upon cortical neurones is identical with that which has been found for spinal motoneurones (Curtis, Phillis and Watkins, 1959), the depression would be explicable by the production of an increase in the conductance of the postsynaptic membrane which reduces the effectiveness of depolarizing currents. It might then be expected that the antidromic invasion of Betz cells could be depressed, as was found in the experiments of Section IV(c) below.

(b) Neutral amino acid actions on cerebral cortical neurones

(i) γ-amino-n-butyric acid (GABA) was a potent depressant of cortical neurones, and in many cases the diffusion of this substance from a pipette containing a 2 M solution (pH = 3.1; pK_1 = 4.23) was sufficient to reduce or even abolish DLH-induced firing rates of over 40/second (Fig. 9A). The depression of excitability produced by GABA was characterized by a short latency of both onset and offset, following the commencement and termination of the ejecting currents within 0.5-2 seconds in almost every case. The spontaneous firing of cortical neurones was also reduced by the ejection of this compound.

The depressant action of GABA could be demonstrated when this amino acid was ejected electrophoretically from solutions within the range of pH 2.9 to pH 7.2. It is thus unlikely that there is any difference between the actions of GABA cations and zwitterions, as was proposed by Kuno (1960, 1961), Muneoka (1961) and Kuno and Muneoka (1961, 1962) (see also Curtis, Phillis and Watkins, 1959; Curtis and Watkins, 1960a, b; Krnjević and Phillis, 1963a).

(ii) γ-amino-β-hydroxybutyric acid (GABOB) was approximately half as potent as GABA as a depressant of the DLH-induced and spontaneous firing of cortical neurones.

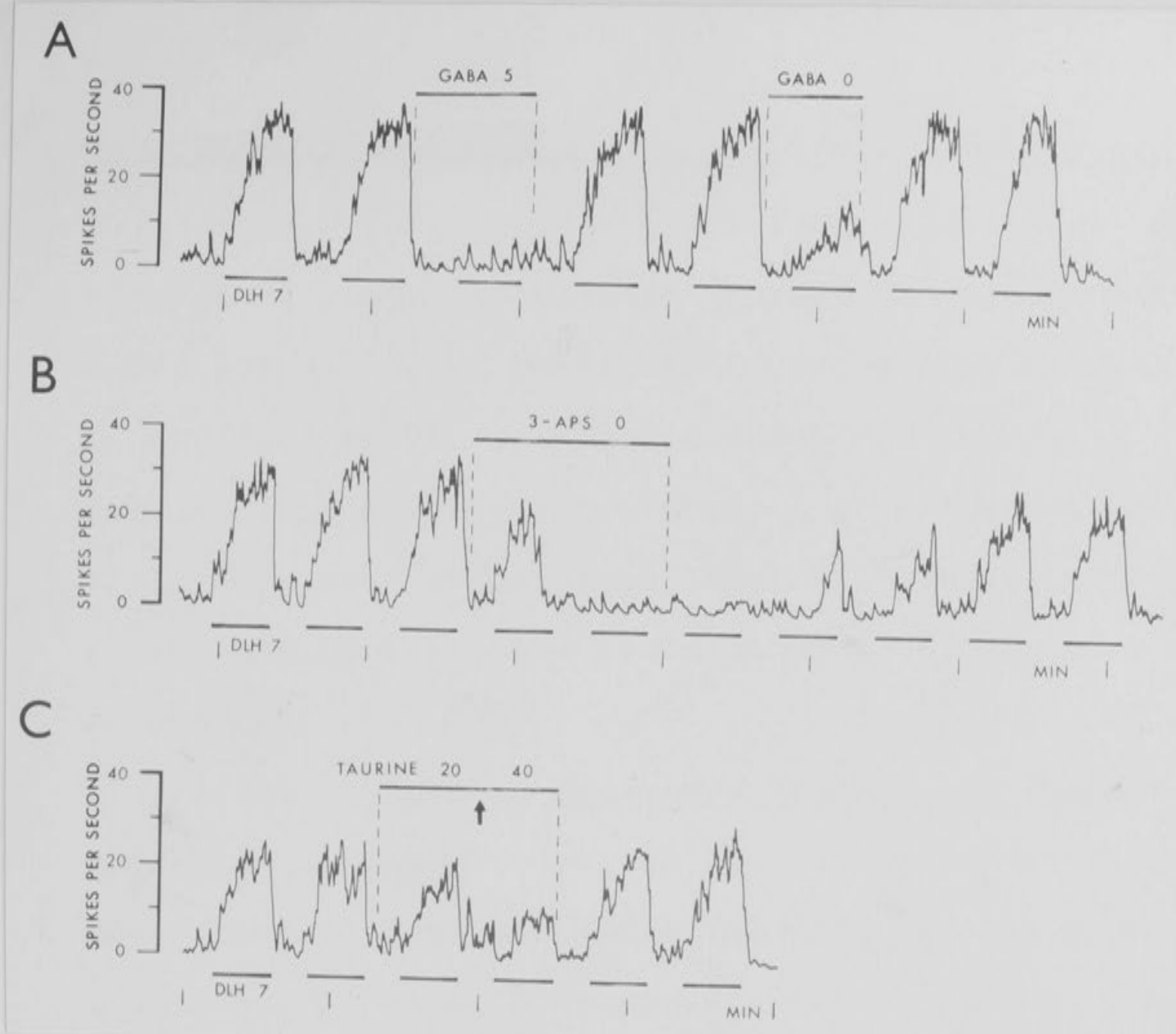

Fig. 9: Interaction of depressant amino acids with the DLH-excitation of a Betz cell.

A: Effect of diffusion (GABA 0) and ejection (GABA 5nA) of γ-amino-n-butyric acid from a 2 M solution, upon the excitation of a Betz cell by DLH ejected with current of 7 nA (lower bars).

B: Effect of diffusion (3APS 0) of 3-aminopropane-1-sulphonic acid from a 0.1 M solution upon the DLH-induced firing of the same unit as in "A".

C: Effect of taurine ejected with a current of 20 and 40 nA upon the DLH excitation of the same unit as in "A" and "B".

Ordinate: Firing frequency in spikes per second.

Abscissa: Time scale in minutes.

This potency difference is similar to that observed in the spinal cord (Curtis and Watkins, 1960b), but contrasts with the findings of Hayashi (1959b) who reported that GABOB was much more powerful than GABA as a depressant of cortical responses when administered topically. Rech and Domino (1960), using topical application of the amino acid to reverse strychnine spikes in the isolated cerebral cortex of the dog, also found GABOB to be the more potent compound.

(iii) 3-aminopropane-1-sulphonic acid (3APS) was sufficiently potent to block the DLH-induced firing of many cortical neurones merely by diffusion from 0.1 M. solutions. Indeed, considerable difficulty was experienced in controlling the diffusional efflux of this amino acid from Pyrex micropipettes, presumably because of the high electro-osmotic mobility of this substance in aqueous solution. 3APS could be ejected either as a cation or an anion from 0.1-0.2 M. solutions of pH 9-10, although its escape by diffusion from the pipette orifice (of about 1μ internal diameter) was checked to a large extent by a small anionic retaining current. When ejected as a cation, 3APS proved to be an extremely powerful depressant of all cortical neurones upon which it was tried. This amino acid exceeded GABA in potency and in the duration of

its action. In Fig. 9B, diffusion of 3-aminopropane-sulphonic acid from a 0.1 M solution (3APS 0) depressed the firing of a cortical neurone to a greater extent than did diffusion of GABA from 2 M solution (GABA 0 in Fig. 9A). As the ejections of both amino acids (and of taurine in Fig. 9C) were from different barrels of the same microelectrode, the internal diameters of the respective micropipette orifices may be assumed to be comparable. The contribution made by diffusion of each amino acid ought then to be proportional to the concentration of its solution. As the potency is inversely proportional to the total dose required to abolish the firing of the cell, it is evident that 3APS is much more potent than either GABA or taurine, although quantitative estimations are impossible to make as the contributions made by bulk flow and electro-osmosis are unknown.

Similar results have been obtained using spinal interneurones as test objects. In earlier experiments, Curtis and Watkins (1961) had used solutions of pH 2.8 for ejecting neutral amino acids as cations in an attempt to overcome the difficulties of retention of these compounds within the micropipettes at pH 9-10 (see above), and under these circumstances also the action of 3APS was greater and more prolonged than that of GABA.

Whenever the diffusional efflux of 3APS could not be adequately controlled (as with the use of more concentrated solutions than 0.2 M, electrodes with internal diameters of the tip orifices of some 2μ, or electrodes which had been stored for more than a few hours, even under nitrogen), very few neurones could be located when these electrodes were passed through cortical or spinal tissue. Not even the intermittent ejection of DLH sufficed to excite cells under these circumstances.

(iv) Taurine was readily ejected as an anion from solutions of pH above 8.5. When ejected with currents of 20-80 nA, taurine depressed or abolished the response to DLH of all cells which were tested (see Fig.9C), and although substantially weaker than either 3APS or GABA, this sulphonic amino acid had an action upon cortical neurones similar to that already reported for neurones in the spinal cord (Curtis, Phillis and Watkins, 1959; Curtis and Watkins, 1960a, 1960b). The action of taurine persisted for 10-15 seconds after the end of the ejection, a duration intermediate between those of GABA and 3-aminopropanesulphonic acid.

(v) Guanidino-acetic acid (glycocyamine) and β-guanidinopropionic acid were ejected as cations from solutions of pH 3. Both guanidino acids depressed the firing of cortical neurones evoked by DL-homocysteic acid

but were less active in this respect than GABA; guanidinopropionic acid was slightly more active than glycocyamine. These substances were not tested as depressants of the synaptic responses of cortical neurones, but in experiments upon spinal interneurones, β-guanidino-propionic acid was found to be the more potent depressant, as it reduced both the synaptic and amino acid induced firing of these cells, whereas guanidino-acetic acid merely reduced the frequency of firing produced by concurrent administration of an excitant amino acid, and failed to affect synaptic excitation (cf. Curtis and Watkins, 1960b). This would seem to indicate that glycocyamine may be able to interfere with the access of excitant amino acids to the membrane receptors, but not to prevent the synaptic depolarization of these cells, nor to hyperpolarize them to a sufficient degree to inhibit their firing.

(vi) ε-aminocaproic acid and ω-aminocaprylic acid were tested as representatives of the long-chain ω-amino acids. Interest in these compounds stems from Hayashi (1956) who suggested that the ω-amino-monocarboxylic acids had both excitatory and inhibitory actions, excitation gradually predominating as the chain length was increased. Purpura and Grundfest and their collaborators (e.g. Purpura, Girado and Grundfest, 1957a, b; Purpura,

Girado, Smith, Callan and Grundfest, 1959) have produced an extensive series of observations on the effects of topical application of these compounds on evoked cortical potentials, and conclude that whereas short chain ω-amino acids block excitatory synapses, the longer-chain compounds preferentially block inhibitory synapses. The results obtained using microelectrophoretic ejection near cortical neurones are not in complete accord with these proposals, however (see also Section IV(f) below).

In agreement with Krnjević and Phillis (1963a), the actions of both ε-aminocaproic acid and ω-aminocaprylic acid were 'somewhat ambiguous' when ejected from solutions of pH 3. This low pH value was used in an attempt to increase the degree of ionization of the amino acids and thus to increase the conductance of the solutions and permit larger electrophoretic currents to be used. Although ε-aminocaproic acid had a slight but definite short-latency depressant action upon cortical neurones, both amino acids eventually produced a delayed high-frequency firing, often accompanied by spike changes, similar to that produced by asparagine (see Fig. 10). The latency of this paroxysmal firing varied from about 15 to 90 seconds depending upon the magnitude of the ejecting current, and firing frequently continued for up to 15 seconds after the cessation of current flow. Subsequently the cell was

quiescent and had a reduced sensitivity to excitant amino acids for a variable period.

Results similar to these have been observed in the spinal cord, but were attributed to local pH changes (Curtis and Watkins, 1960b, page 119). Both spinal interneurones and cortical neurones are sensitive to local pH changes (Curtis, Phillis and Watkins, 1959, 1961; Krnjević and Phillis, 1963a; Krnjević, 1964, 1965a) and although the sensitivity of different cells varies considerably, it is unusual to be able to demonstrate such an action when using acidified NaCl solutions of pH greater than 2.5. However, neurones are readily excited when a cationic current is passed through such solutions of pH less than 2, and then give paroxysmal firing of the type produced by acidic solutions of ω-aminocaprylic acid or asparagine (see Section IV(c) below). When cationic currents are passed through low-pH solutions of weak bases or doubly ionized compounds, the release of hydrogen ion is greater than that obtained if the pipette contained a NaCl solution of the same pH. Each amino acid cation would release its proton on reaching the external medium, and the change in local pH around the pipette orifice approximates that obtained by passing the current through a solution of hydrogen chloride of the same concentration (0.1-2 M) as the amino acid, although the solution within

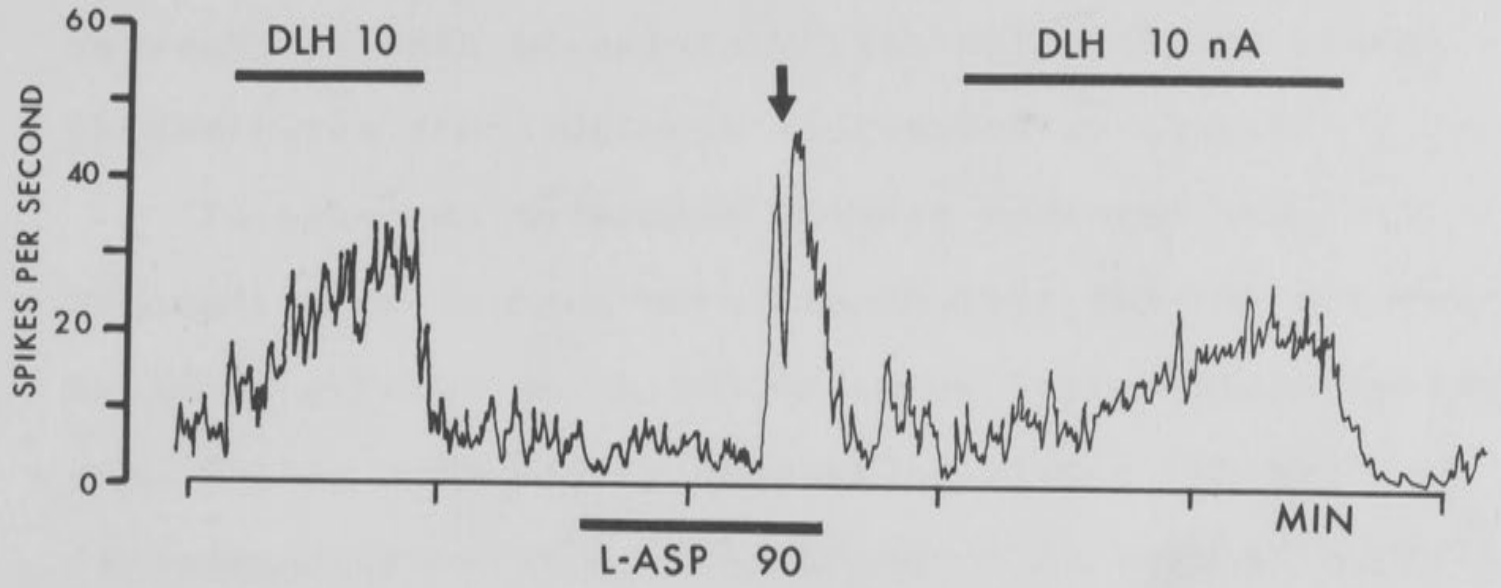

Fig. 10: Firing frequency of a Betz cell excited by ejection of DL-homocysteic acid (current of 10 nA), and by ejection of L-asparagine from a solution of pH = 3.1 (L-ASP, 90 nA). After 66 seconds of the latter, the cell began to fire rapidly but the spikes soon deteriorated in size and became predominantly positive (arrow). The sensitivity of the cell to the acidic amino acid was thereafter reduced for a period of over $6\frac{1}{2}$ minutes.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time scale (minutes).

the pipette contains only sufficient free hydrogen ions to maintain a pH of 3-4. When the ejected amino acids are sufficiently potent neuronal depressants (as with the short-chain ω-amino acids), the excitation by hydrogen ion is overcome by the membrane conductance change induced in the cell. On the other hand if the depressant action is weak, as with ω-aminocaprylic acid, the pH change predominates and a delayed excitation is seen.

In order to determine whether hydrogen ion concentration changes were responsible for the delayed excitant effects of these ω-amino acids, micropipettes were filled with nearly neutral solutions (pH 6.7 for ε-aminocaproic acid; ω-aminocaprylic acid at pH 7.5), and the amino acid solutions ejected by hydrostatic pressure applied to the upper end of the drug barrels, as electrophoretic currents were limited to less than 10-20 nA by the high resistance of these solutions. Under these conditions, using pressures of the order of 300 mm. Hg for as long as 3 minutes, no excitation was ever noted.

It was not possible to prove that satisfactory microinjection by this technique had been achieved, but considerably lower pressures sufficed to eject DLH or GABA from other barrels of the same microelectrode, as was shown by excitation and depression respectively of the

neighbouring cells. ϵ-aminocaproic acid usually reduced the DLH-evoked firing of cortical neurones when ejected by pressure in this manner, although neutral ω-aminocaprylic acid appeared inactive.

(c) Amides and other compounds related to the neutral amino acids

(i) D- and L-asparagine. The only amides of acidic amino acids which were tested were the D- and L-isomers of asparagine (Table III). Except when ejected electrophoretically from solutions of pH 3-4, both of these substances were inert, whether ejected by pressure (300 mm. Hg) or by microelectrophoresis (currents up to 30 nA). Larger currents (50-90 nA) through solutions of L-asparagine of pH 3 caused a delayed paroxysmal firing of cortical neurones in which the spikes frequently became positive (Fig. 10). The latency of this excitation was of the order of 20-90 seconds, and the paroxysm lasted for several seconds after the administration had ceased. The cell was subsequently less sensitive to test doses of DLH for up to 3-4 minutes, and a few cells showed irreversible damage attributed to the local pH changes. A similar delayed firing of spinal interneurones was attributed by Curtis and Watkins (1960b) to the effects of hydrogen ion rather than to an action of the amides themselves.

(ii) Sodium 4-hydroxybutyrate. Although not an amino acid, this compound was of interest because of its depressant activity and synergism with other depressants when given systemically (Laborit, Jouany, Gerard and Fabiani, 1960; Laborit, Kind and Regil, 1961; Drakonatides, Schneider and Funderburk, 1962; Laborit, 1964). Basil, Blair and Holmes (1964) have also found it to depress spinal reflexes after systemic administration. However, electrophoretic ejection of this compound (using currents as great as 100 nA) failed to modify either the spontaneous activity of cortical neurones, or the spikes evoked by an excitant amino acid. Spinal interneurones behaved similarly towards this agent - no demonstrable depressant activity was noted. In view of these negative findings and the long latency of its action following intravenous administration, γ-hydroxybutyrate is probably acting indirectly, possibly by interfering with the metabolism of GABA (cf. Drakontides et al, 1962; Basil et al, 1964).

(iii) γ-aminobutyrylcholine. The available sample of this compound (California Corporation for Biochemical Research) contained γ-aminobutyric acid, and the observed weak depressant action upon the spontaneous and evoked responses of cortical neurones was attributable in large part to this contaminant.

(d) Effect of neutral amino acids upon the antidromic invasion of Betz cells

Even in the absence of spontaneous firing the safety factor for the antidromic invasion of the body and dendrites of Betz cells is high (see Phillips, 1959) and it was anticipated that some difficulty would be encountered in attempts to test the effect of depressant amino acids upon this form of firing. Krnjević and Phillis (1963a) and Krnjević (1964) also report that antidromic invasion of Betz cells was not readily blocked by GABA. In the present series, currents of the order of 100-200 nA ejecting GABA were found to block antidromic firing, causing either partial fragmentation of the soma-dendritic spike to a smaller 'IS' spike (Eccles, 1957, pp.48-49) or complete suppression of the SD spike to leave a small axonal or M spike. The depression of these antidromic responses was readily measured by the 'firing index', which expressed the number of SD spikes as a percentage of responses in a series of ten supramaximal axonal stimuli. The repetition rate of stimulation was 1 or 2 stimuli per second in these experiments.

3-aminopropanesulphonic acid was more effective than GABA as a depressant of antidromic invasion of Betz cells. The action of this amino acid upon the antidromic spikes of one cell is illustrated in Fig. 11. The stimulus

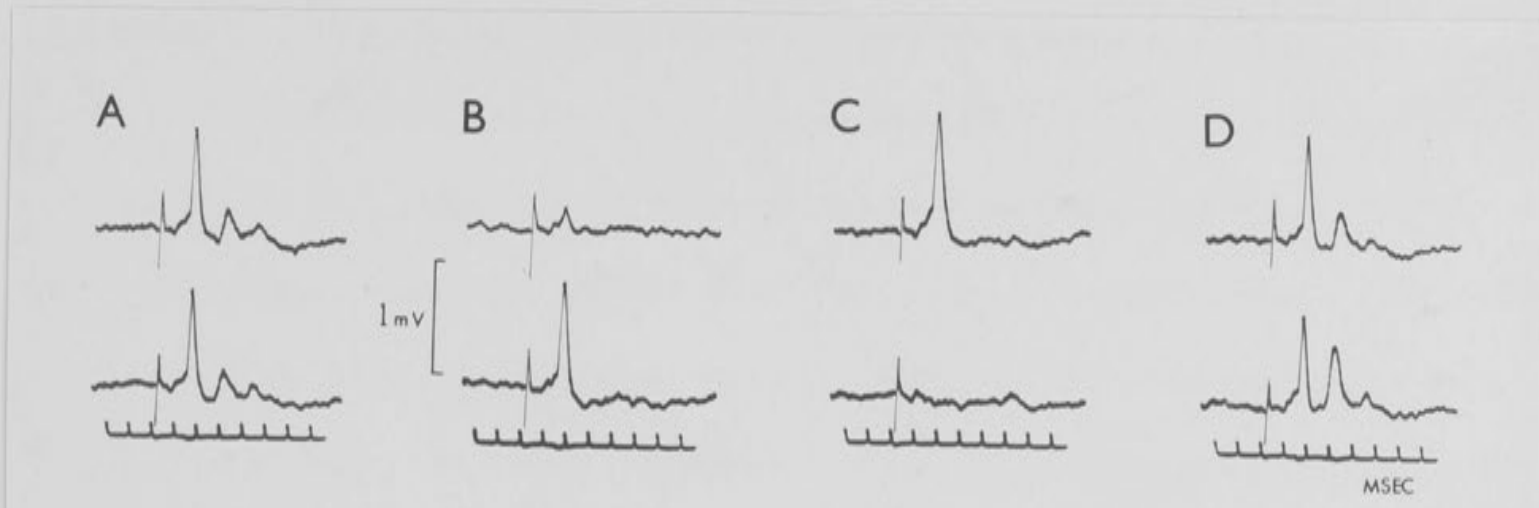

Fig. 11: Depression of antidromic invasion of a Betz cell by the ejection of 3-aminopropane-sulphonic acid.

A: Control-firing index 100%.

B: 10 Seconds after start of 3APS ejection (current of 80 nA) - firing index 80%.

C: 6 Seconds after 3APS current increased to 150 nA - firing index 50%.

D: 10 Seconds after end of 3APS ejection - firing index 100%. For further description, see text.

Time scale: Msec, all traces.

Negativity recorded upwards.

strength applied to the pyramids was supramaximal for the axon of this cell, and remained constant during the series. The antidromic spike potential had a latency of 1.27 msec., and was followed by a complex field potential produced by the antidromic and synaptic excitation of other neighbouring neurones. The firing index of this cell was initially 100 per cent (A), but fell to 80 per cent within ten seconds of the ejection of 3APS with a cationic current of 80 nA (B). When the electrophoretic current was increased to 150 nA the firing index fell to 50 per cent within 5-10 seconds (C). Recovery of the firing index to 100 per cent occurred within a few seconds of the termination of the current (D), and simultaneously the field potentials which had been virtually abolished during the ejection of 3APS also recovered.

(e) Intraventricular and intracerebral injection in mice

Three of the more potent depressant amino acids were also tested by intraventricular injection in mice. These were 3-aminopropane-1-sulphonic acid, GABA and taurine. As with the excitant (acidic) series described in Section III(e), the drugs were injected in 0.05 ml. volumes of buffed physiological saline, according to the method of Haley and McCormick (1957).

Doses of 100 µg of 3APS, or 150-250 µg of GABA, produced loss of muscle tone and gross inco-ordination of movement. The animals were unable to right themselves when placed upon their back or sides, and after a few minutes in this position abandoned the attempt and appeared to fall asleep. They could readily be alerted by movement or stroking the fur, but made no effort to evade the stimuli, and relapsed into the sleep-like state soon after stimulation ceased. Gradual recovery over a period of ½-2 hours was the rule. In one experiment using 200 µg doses of 3-aminopropanesulphonic acid, two of the group of four mice died with respiratory failure, but no systematic attempt was made to determine lethal dosages of the depressant amino acids. Uncertainty of the site of action in these experiments would render such studies virtually useless in their present form.

Smaller doses of these amino acids also produced inco-ordination of movement and transient flaccidity of the limbs and body. Gradual recovery usually took about one hour.

In these experiments, somewhat larger doses of taurine than of GABA or 3APS were required for comparable effects. Thus the depressant amino acids tested showed an order of potency comparable to that determined by electrophoretic ejection upon single neurones, although taurine appeared

rather more active (only slightly less potent than GABA) than might have been predicted. The results are summarized in Table IV.

(f) Summary and discussion of excitant and depressant amino acid actions

Many amino acids have been administered topically in solution to the exposed cerebral cortex (Hayashi, 1954, 1959a, b; Iwama and Jasper, 1957; Purpura, Girado, Smith, Callan and Grundfest, 1959; Curtis and Watkins, 1961) and although it has been suggested that some of these substances have specific effects at particular synapses (Purpura, Girado and Grundfest, 1957, 1958; Purpura et al, 1959; Purpura, 1959, 1960; cf. Grundfest, 1964), this proposal has met with certain difficulties (e.g. Jasper, 1960). These difficulties include the interpretation of changes in the potentials recorded from the surface of cortical tissue (see also Rech and Domino, 1960), and experiments in which topical administration of amino acids has been combined with recording of activity at different depths beneath the surface (Bindman, Lippold and Redfearn, 1962) have failed to confirm the hypothesis that GABA is a selective inactivator of the depolarizing excitatory synapses of superficial dendrites (Purpura et al, 1957, 1958, 1959). Krnjević (1964) was unable to

demonstrate hyperpolarization of cortical neurones, an alternative proposal for the mode of action of this depressant.

The results obtained with extracellular ejection and recording of activity of cortical neurones are in many respects concordant with those obtained by topical administration of the amino acids, or intravenous presentation in cases in which the blood-brain barrier does not hinder entry of the drug in to cortical tissue. Thus, short-chain ω-amino acids depress cortical neurones, whereas many acidic amino acids produce an excitation which, if excessive, leads to spreading depression (Hayashi, 1954, 1956, 1959; Purpura et al, 1957, 1958, 1959; Iwama and Jasper, 1957; Van Harreveld, 1959; Bindman et al, 1962; Curtis and Watkins, 1961, 1963, 1965; Krnjević and Phillis, 1963a).

The importance of the present results lies in the demonstration of the close similarity between the responses of all cortical neurones, including Betz cells, to those of neurones elsewhere in the feline nervous system when tested with the amino acids by close electrophoretic ejection (see references cited in introduction to Section III, above). This pattern of uniform amino acid sensitivity is in marked contrast to the behaviour of neurones in various regions towards acetylcholine,

cholinomimetics and acetylcholine antagonists, which will be discussed in later Sections, or towards indole and phenylethylamine derivatives.

The differences between the results presented in Krnjević and Phillis (1963a) and the present series were attributed in large part to differences in technique, in particular the use of a ratemeter and paper recorder to ensure that the peak effect of a given dose of an amino acid had been reached before the ejection was terminated. In this manner a ready comparison can be made of the relative potencies of substances with different rates of action, such as N-methyl-D-aspartic acid and L-glutamate. The different assessment of the relative potencies of the insomers of glutamic acid by Krnjević and Phillis (1961, 1963a) appears to be due to their comparison of the effects produced by equal concentrations (currents) rather than of the currents required to produce equal effects (see Section III(b) above, and Krnjević and Phillis, 1963a, p.282). It cannot be agreed that D-glutamate was 'much less effective' than the L-isomer, as the potency ratio by the method of Section III(b) is 0.5 to 0.8.

One interesting facet of amino acid action upon cortical (and spinal) neurones is the depression in spontaneous firing which is observed to follow excitation by the acidic amino acids, particularly DL-homocysteic

acid and N-methyl-D-aspartic. Such a depression is observed in virtually all spontaneously active units, and is more pronounced and of longer duration when the cell has been intensively activated by the excitant. However, it is not due to the production of a depolarization block, as it may occur without any evidence of changes in spike shape. The phenomenon may represent a 'non-specific desensitization' of the cell by the amino acid to synaptic transmitters (cf. Curtis and Ryall, 1965d; Paton, 1961), or may reflect depolarization of the presynaptic terminals impinging on the cell, with consequent diminution of transmitter output (see Eccles, 1963; Curtis and Ryall, 1965d). In contrast, the desensitization of cortical and spinal neurones by one amino acid to another is dependent upon the production of 'depolarization block' by one of the excitants (Section III(b)i; Figs. 6 and 7), and is not due to a specific interference of access to the receptor sites (Krnjević and Phillis, 1963a, page 299). Recovery of the amino acid sensitivity after such a 'block', however, is incomplete until well after the restoration of antidromic invasion and the normal spike shape and amplitude.

The weak depressant actions reported by Krnjević and Phillis (1961, 1963a) for electrophoretically administered taurine and 3-aminopropanesulphonic acid are rather

surprising since, like GABA, both of these sulphonic amino acids readily depress cortical responses when applied topically in solution to the exposed surface (Curtis and Watkins, 1961; but see also Purpura et al., 1959). Furthermore, when injected intraventricularly into mice, all three compounds result in a diminution of spontaneous activity and eventually in a state resembling sleep (Section IV(e) above). Again, the relative potencies of the amino acids were similar to those observed in electrophoretic studies upon single cortical and spinal neurones.

In view of the inability to reproduce the delayed excitation by ω-aminocaprylic acid or asparagine when these substances were ejected hydrostatically from neutral solutions, it cannot be stated that these compounds excite neurones, nor that the results obtained electrophoretically are in agreement with those of Hayashi (1956), Purpura et al. (1959) or van Harreveld (1959), who administered the solutions topically to the exposed cortex. On the other hand, if indeed the compounds to excite cortical neurones other than by induced pH changes (Sections IV(b)vi and IV(c)i above), then their effects are still identical with those upon spinal neurones, as Curtis and Watkins (1960b) in their earlier experiments had considered that the delayed excitation could not be directly associated with an action of the amino acid molecule itself.

The failure of γ-hydroxybutyric acid to affect central neurones, either cortical or spinal, suggests that the alterations in behaviour, in the electroncephalogram and in spinal reflexes which are produced by this substance are unlikely to be due to a direct effect upon central cells (see Laborit et al., 1960, 1961; Drakontides et al., 1962; Basil et al., 1964) but possibly rather to some alteration of glutamate or GABA metabolism (Laborit, 1964). It is pertinent that a related substance, γ-butyrolactone, which also has central depressant effects (Giarman and Schmidt, 1963) does not increase the brain level of GABA. However, the total brain concentration of GABA does not necessarily correlate with the excitability of central neurones or the overall behavioural state of the animal as indicated by convulsions, etc. (for example, see Kamrin and Kamrin, 1961; Medina, 1963; Sytinskii and Priyatkina, 1964). It may well be necessary to ascertain differences in the distribution of amino acids between the intra- and extra-cellular compartments of the c.n.s. (see also Curtis and Watkins, 1960a, 1965).

The relevance of these electrophoretic studies to the distribution of L-glutamic acid in nervous tissue, to the disturbances of amino acid levels in convulsive states, and to the phenomenon of spreading cortical depression are discussed elsewhere (e.g. van Harreveld, 1959; Curtis,

Phillis and Watkins, 1960; Curtis and Watkins, 1960a) and have been fully reviewed by Curtis and Watkins (1965).

As predicted from the behaviour of spinal motoneurones, GABA and more particularly 3-aminopropanesulphonic acid were able to block the antidromic invasion of Betz cells by their production of an increased membrane conductance, the difficulty which was encountered in performing this being a reflection of the high safety factor of this type of excitation and of the limited portion of the cell membrane which was affected by the local administration of the amino acid. These findings do not support the hypothesis of Purpura and Grundfest and their co-workers (Purpura et al., 1957, 1959; Purpura, 1960; Grundfest, 1958, 1960, 1964) that GABA and other short-chain ω-amino acids act specifically to block excitatory axodendritic synapses.

An alternative proposal (e.g. Krnjević, Randić and Straughan, 1964; Krnjević, 1964, 1965a) that GABA may be a central inhibitory transmitter appears unproven at present because this substance fails to hyperpolarize cortical neurones (Krnjević and Phillis, unpublished, cited by Krnjević, 1964). Intracellular recording techniques had earlier been employed with spinal motoneurones (Curtis, Phillis and Watkins, 1959), using extracellular ejection of GABA and β-alanine. Despite

impressive depressant actions upon the firing of both cortical and spinal neurones, these neutral amino acids do not appreciably alter the membrane resting potential - in other words, their actions are not those to be expected of an inhibitory transmitter. For other cell types, from which intracellular records have yet to be obtained, the argument against an inhibitory transmitter role must proceed by analogy, as extracellular recording of action potentials yields no information as to possible membrane potential changes associated with periods of depressed firing.

Using intracellular recording from spinal motoneurones, Curtis (1962a, 1965) has also shown that the equilibrium potential for the depolarization due to extracellular ejection of L-glutamate differs from that of the excitatory postsynaptic potential. Reversal of the amino acid induced depolarization occurs at a more polarized level than does that for synaptic excitation, and this difference has been attributed to involvement of conductance changes for different ions in the two cases (see also Curtis, Phillis and Watkins, 1960; Eccles, 1964, chapter IV; Curtis and Watkins, 1965).

Further indirect evidence of the involvement of amino acids in the functional activity of neurones has recently been supplied by the findings of Elliott, Khan

and Jasper (1964) that L-glutamate and GABA are released from the cerebral cortex at rates dependent upon the state of activation as indicated by the electroencephalogram. Under conditions of EEG arousal glutamate release is predominant, whereas with a 'sleeping' EEG more GABA is released. The release of L-aspartate is not correlated with the EEG pattern, rendering unlikely general effects on amino acid metabolism or changes in the cortical blood flow. However, release of these amino acids from presynaptic neuronal terminals has not been established and it is perhaps relevant that Ryall (1962, 1964) and Weinstein, Roberts and Kakefuda (1963) have failed to find any special concentration of these acids in the synaptic-ending fractions of homogenised brain, as might be expected for transmitter substances (Section I, criterion (b)). Acetylcholine, for instance, has been shown to be associated with synaptic vesicles in the nerve-ending fractions of similar homogenates by many workers, including Hebb and Whittaker, (1958), de Robertis, Pellegrino de Iraldi, Rodriguez de Lorez Arnaiz and Salganicoff (1962), de Robertis, Rodriguez de Lorez Arnaiz, Salganicoff, Pellegrino de Iraldi and Zieher (1963), and Whittaker, Michaelson and Kirkland (1963, 1964) as well as by Ryall (1964).

It is perhaps advisable for the present to state that a transmitter function for the amino acids in the central nervous system is unlikely, although far from being disproven. The remarkable similarities in potency and time-course of action for a given amino acid on all types of mammalian central neurone so far examined suggests that essentially similar receptors exist on all nerve cells, (see Curtis and Watkins, 1960b, 1963, 1965; Watkins, 1965). A 'two-point' or 'three-point' receptor of the type envisaged by Curtis and Watkins (1960b, 1963) for spinal neurones would equally well explain the results observed with cortical neurones in the present experiments. In his most recent extension of this theory, Watkins (1965) suggests that the receptors with which the amino acids combine are areas of phospholipid constructional material incorporating constituent organic bases structurally similar to the amino acids.

SECTION V - ACETYLCHOLINE

(a) Introduction

Considerable indirect evidence as to the possible function of acetylcholine (ACh) as a transmitter substance in the higher regions of the central nervous system is presented in reviews by Feldberg (1945, 1957) and Hebb (1957). Interest has been further stimulated by the reports that electrophoretically administered ACh excites neurones in the cerebral cortex (Spehlmann and Kapp, 1961; Krnjević and Phillis, 1961b, 1962, 1963a, 1963b, c; Spehlmann, 1963; Salmoiraghi and Stefanis 1965), cerebellar cortex (Krnjević and Phillis, 1963a; McCance and Phillis, 1964a, b), hippocampus (Stefanis, 1964; Salmoiraghi and Stefanis 1965; Biscoe and Straughan, 1965), thalamus (Curtis and Andersen, 1962; Andersen and Curtis, 1964a, b) and brain stem and reticular formation (Curtis and Koizumi, 1961; Bradley and Wolstencroft, 1962; Salmoiraghi and Steiner, 1963). Complementary to these reports have been the recent histochemical determinations of the distribution of acetylcholinesterase (AChE) in the cerebral (Krnjević and Silver, 1963a, b; Krnjević, 1965b), cerebellar (Sperti, Sperti and Zatti, 1960; Snell, 1961; Austin, Phillis and Steele, 1964; Freide and Fleming, 1964) and

hippocampal cortices (Lewis, Shute and Silver, 1964), while similar studies on brain-stem afferent systems (Shute and Lewis, 1961, 1963a, b, 1965) suggest a possible source of cholinergic innervation of these structures from the reticular system.

An extensive review of histochemical and allied techniques for cholinesterase estimation, and of the possible physiological roles played by ACh and acetylcholinesterase, has been given by Koelle (1963).

Undercutting of the cerebral cortex produces a decrease in the amounts of AChE and acetyltransferase demonstrable in the isolated areas of the pericruciate and suprasylvian cortex (Hebb, Krnjević and Silver, 1963), and after cholinesterases have been inactivated, it is possible to demonstrate the release of acetylcholine from the cerebral cortex in amounts which correlate with the state of cortical activity (Elliott, Swank and Henderson, 1950; MacIntosh and Oborin, 1953; Mitchell, 1963; Szerb, 1963).

As the technique of microelectrophoresis is still relatively new it was of interest to confirm and extend some of the earlier studies on neurones of the pericruciate cerebral cortex, the pyramidal and granular layer cells of the cerebellar vermis, and pyramidal neurones of the

dorsal hippocampus. In this manner it was hoped to clear up some discrepancies in the actions reported in the literature, and to establish the most suitable type of preparation (particularly with regard to depth of anaesthesia and type of anaesthetic agent used) for a comparison of the pharmacology of the ACh-induced and synaptic excitation of these neurones.

(b) Action upon Betz cells of the pericruciate cortex

Preliminary experiments were made upon 15 cats anaesthetized with pentobarbitone sodium, diallylbarbituric acid, or Dial compound (Ciba Ltd., diallylbarbituric acid plus urethane). Subsequently, a further series of nine cerveau isolé cats was used for a systematic comparison of the presence and behaviour of cholinoceptive pericruciate neurones in unanaesthetized preparations with those of Betz and other (unidentified) neurones in the anaesthetized animal.

Betz cells of the pericruciate region were identified by their responses to stimulation of the ipsilateral medullary pyramid (see Section II(h)). These cells were usually located at depths of 0.7-1.4 mm. beneath the cortical surface, and characteristically showed more or less random firing at rates of between 2 and 20/second, although occasionally higher rates were noted. This random

discharge pattern must be clearly differentiated from the 'spindling' often seen in cortical and thalamic neurones following barbiturate anaesthesia (Verzeano and Calma, 1954; Andersen and Curtis, 1964a; Andersen and Sears, 1964). Cortical neurones which were strongly driven from the thalamus in spindles were often insensitive to excitation by ACh, or required larger doses of this excitant than other randomly-firing cells.

In any one track made by the microelectrode in the pericruciate region, both Betz and non-Betz cells were encountered. The 'superficial' cells, at depths from 0.15 to about 0.6 or 0.7 mm., could not be identified by their responses to pyramidal stimulation, and are classified as non-Betz. Thereafter, both Betz and non-Betz cells were found to be intermingled at depths down to 1.5 mm., the greatest depth of penetration routinely attempted. In a few cases, a neurone was found which gave a response to pyramidal stimulation of more than 5 msec. latency, or which failed to follow repetitive pyramidal stimulation above 500/second. These cells are included in Table V as 'possibly Betz' cells, as in general their other characteristics resemble those of the identified Betz cell population. As each cell was encountered, its response to acidic amino acids and to acetylcholine was noted, in addition to any other features of its behaviour.

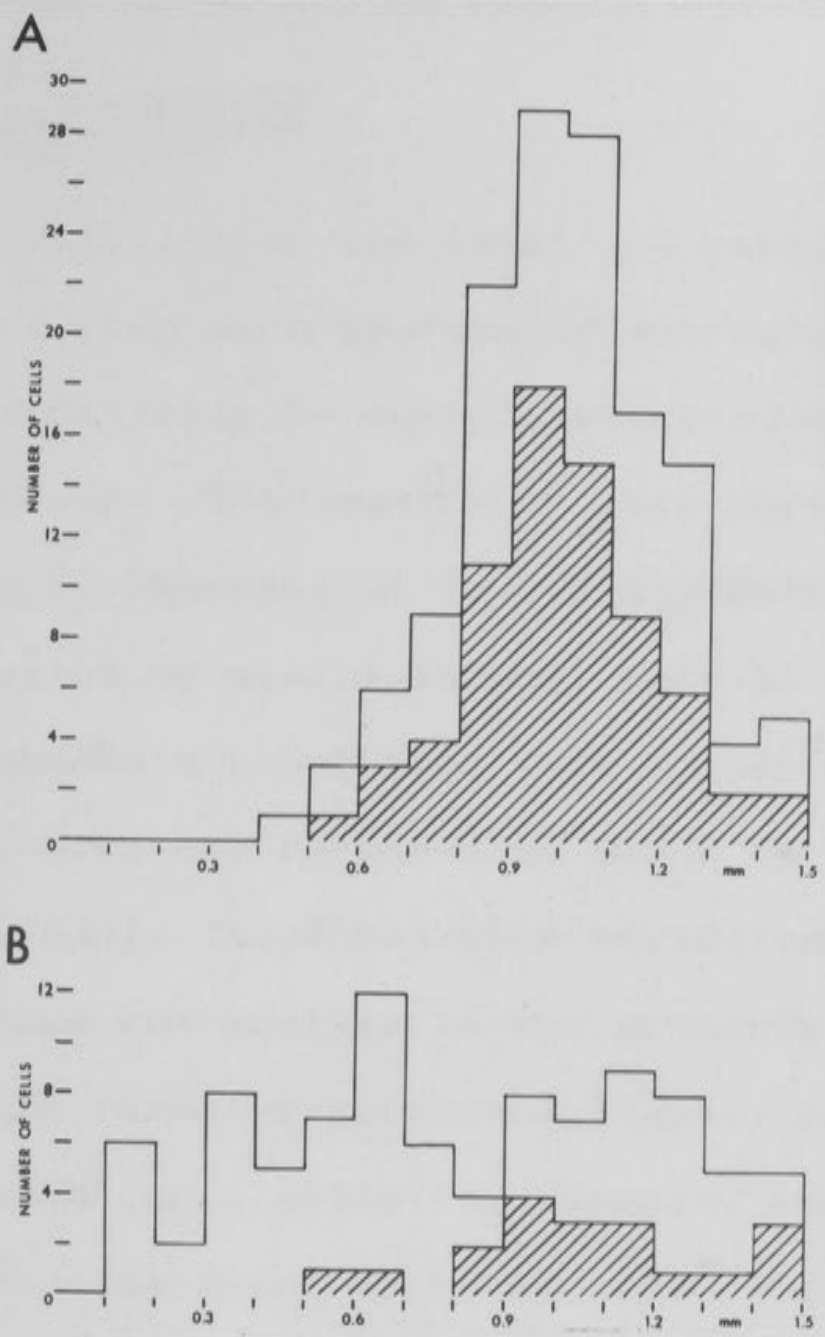

Fig. 12: Depth-distribution of all cells examined in pericruciate gyri of anaesthetised cats. ACh-sensitive members of each population are shown hatched.

A: Pyramidal cells (identified by criteria of Section II (h) i).

B: Non-pyramidal and insufficiently-identified cells.

The class intervals are in 0.1 mm steps.

Ordinate: Number of cells within each class interval.

In all, 231 of the total 236 pericruciate neurones studied during such systematic searches were tested for their sensitivity to acetylcholine ejected with currents of 10-100 nA. The results of this survey are summarized in Table V. Whereas 71 out of 139 Betz cells (51 per cent) were excited by acetylcholine, only 11 of 65 (17 per cent) of the non-Betz population were either fired or facilitated by ACh. However, as was also found by Krnjević and Phillis (1962, 1963b), there was wide variation between one preparation and another in the proportion of cholinoceptive units. In three preparations lightly anaesthetized with diallylbarbituric acid, for example, every Betz cell identified was found to be excited by ACh. Although the depth of anaesthesia and possibly also the particular anaesthetic agent used may affect the proportion of cholinoceptive units encountered (cf. Section X below), these are not the only variable factors - the general condition of the cat, with special reference to the state of its cortical circulation, must also be important. One unanaesthetized cerveau isolé preparation failed to show any cholinoceptive cells, in spite of normal systemic blood pressure, careful surgery and an apparently normal cortical circulatory pattern, and the presence of numerous cells which were excited by DLH.

Table V also emphasizes the tendency of cells which showed a moderate random spontaneous firing to be sensitive to acetylcholine, in agreement with the findings of Krnjević and Phillis (1963b).

In most cases, excitation by ACh was shown by an actual increase in the rate of firing of the cell (e.g. Figs. 13, 20, 21), but in a few cells subliminal excitation only could be demonstrated by the increased firing rate produced by the concurrent ejection of a small amount of an excitant amino acid. The excitation of Betz cells by acetylcholine was characteristically of slow onset, with a latency of 5-15 seconds, and in many cells was preceded by a short-latency transient depression of the firing rate (Fig. 13A, B). Following termination of the ejecting current, the firing usually declined over a period of 15-30 seconds, but occasionally some excitation persisted for as much as a minute (e.g. Fig. 21).

Rarely, the initial depressant action persisted throughout the period of drug ejection and there was no evidence of excitation by acetylcholine until the ejecting current had been terminated, while in other cells ACh caused only depression. In all, 23 neurones were depressed during the passage of ACh, using currents of 20-100 nA. Twelve of these showed a similar depression, with a very

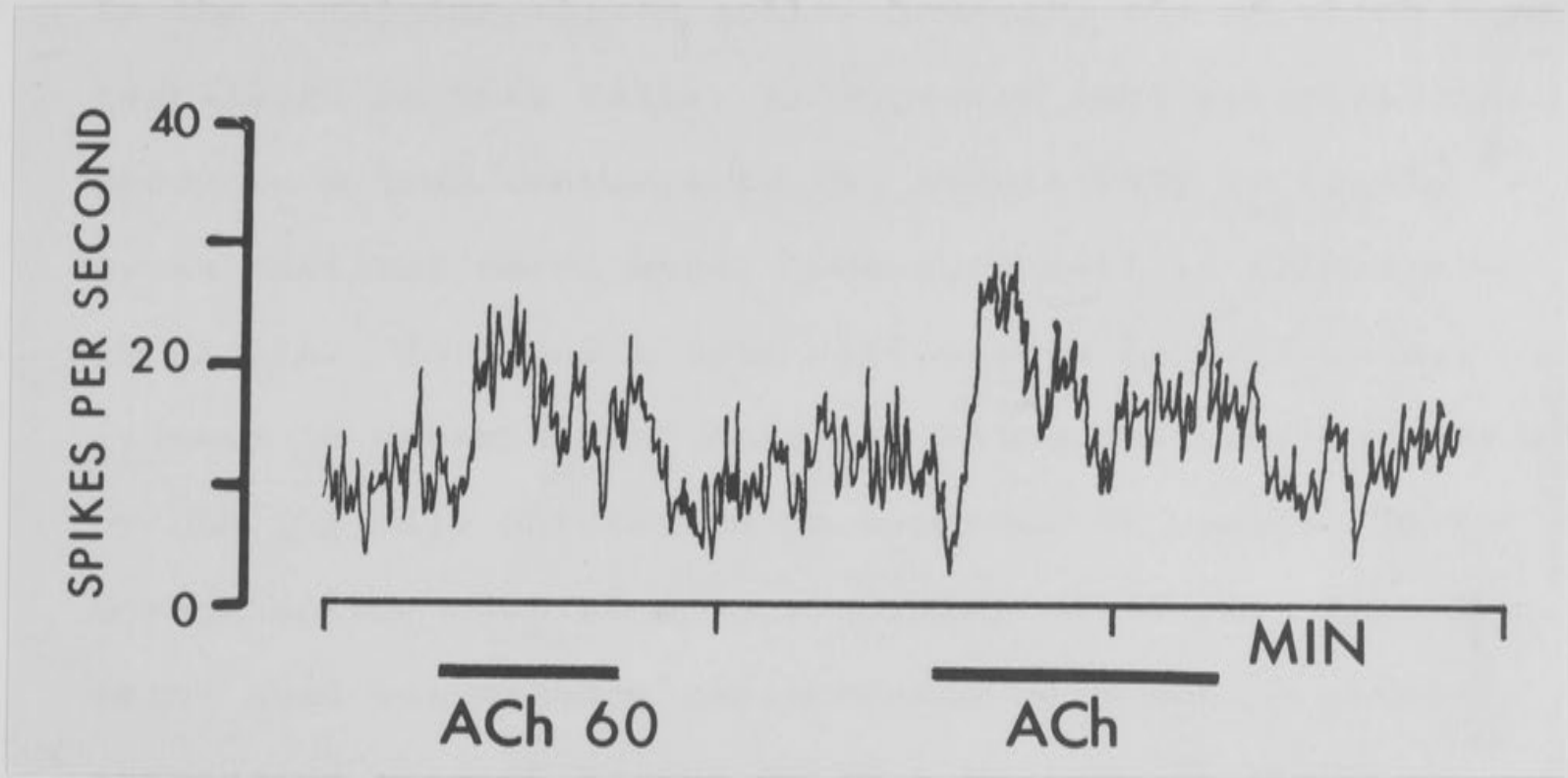

Fig. 13: Firing frequency of a Betz cell excited by acetylcholine (current of 60 nA). Two consecutive trials are shown by the horizontal bars, and demonstrate the variability in response occasionally seen with cortical neurones. In the second trial, the phase of initial depression of the firing rate by acetylcholine is much more pronounced than in the first case.
Time scale in minutes.

rapid onset and offset, when a cationic current was allowed to flow from an adjacent barrel containing NaCl. In the remaining eleven cells, however, six of which were identified as Betz cells, it appeared that acetylcholine produced a true decrease in the sensitivity to firing by an excitant amino acid. One such cell is illustrated in Fig.14. This was a Betz cell with a long (5 msec.) latency to pyramidal stimulation which was readily excited by DLH ($3\frac{1}{2}$ nA), but showed no evidence of excitation by acetylcholine ejected with a current of 40 nA. When the amino acid was ejected concurrently with ACh, a slowly increasing rate of firing up to a maximum of 15-20 spikes per second was observed. The ACh ejection was then terminated, and the sensitivity of the cell to DLH had fully recovered when it was tested twelve seconds after the end of acetylcholine administration. The effect of a cationic current of 60 nA carrying Na^+ ions was merely to increase the rate attainable by DLH without the production of depolarization block, and was therefore opposed to that of ACh.

A similar direct depressant action of ACh probably accounted in part for the transient fall in firing rate which preceded the excitation of many Betz cells by this substance and other cholinomimetics, although the passage of the cationic current also contributes to the phenomenon.

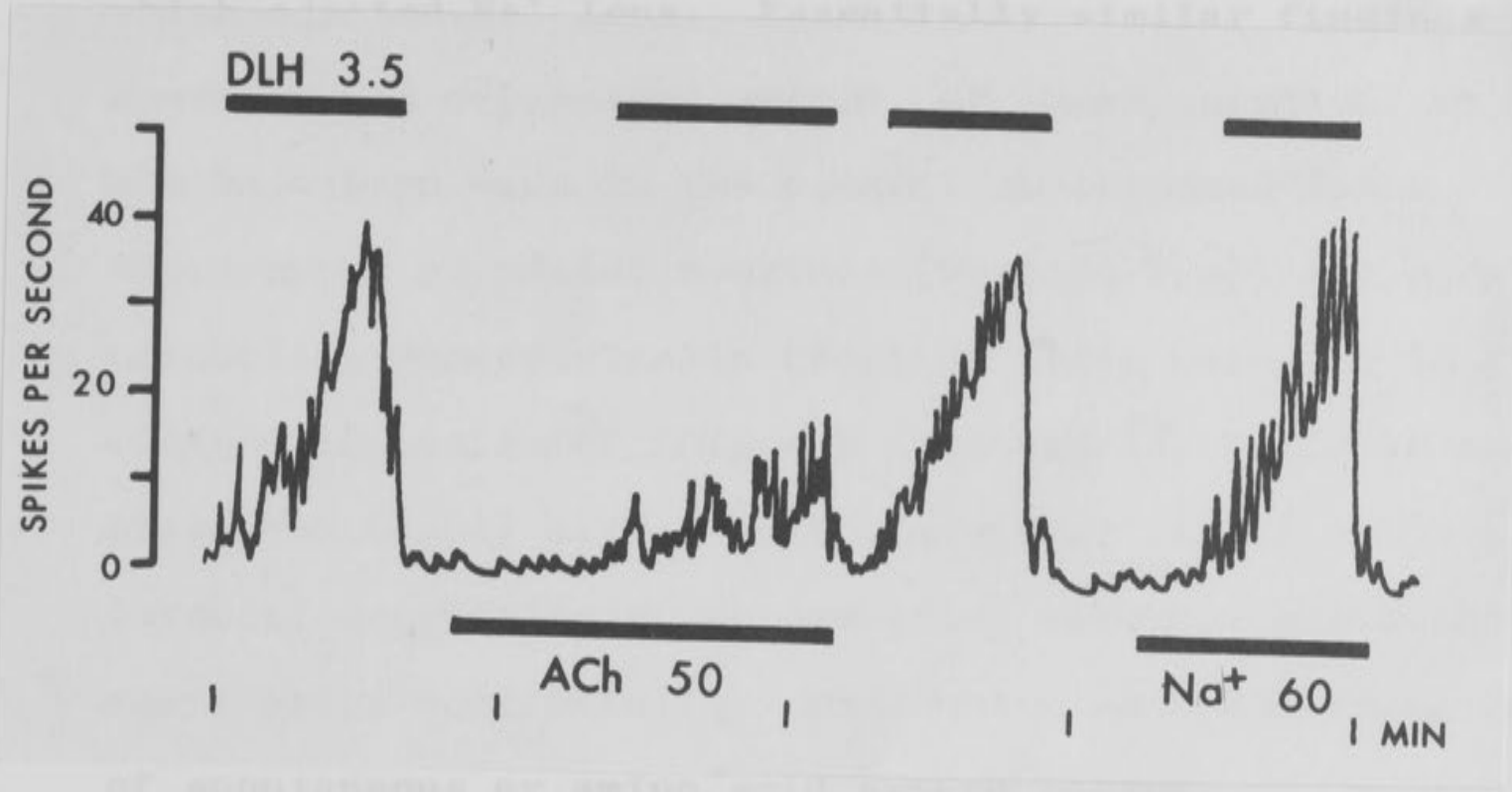

Fig. 14: Depression of DLH-firing of another Betz cell by ejection of acetylcholine (ACh, 40 nA) in the period indicated by the lower horizontal bar. The effect of a cationic current (60 nA) carrying sodium ions is shown in the later part of the figure.

Ordinate: Firing frequency in spikes per second.

Abscissa: Time in minutes. For further description, see text.

The depression had an almost instantaneous onset and offset, conterminous with the drug ejection, but as a rule was only partially imitated by even larger currents which ejected Na^+ ions. Essentially similar findings of a very rapid depressant action, of short duration, of ACh have been made in the present experiments upon hippocampal pyramidal neurones (Section V(e)) and upon cerebellar Purkinje cells (Section V(f), below). In this connection, it is of interest that Randić, Siminoff and Straughan (1964) have reported a slower (10-15 sec. latency) depression of unidentified cerebral cortical neurones by acetylcholine, manifested as a decreased rate of spontaneous or amino acid evoked firing.

(c) Action upon other cerebral cortical neurones

These results are also included in Table V (see above) as they were obtained concurrently with those of the Betz cell population. In general, the 'superficial' group of neurones which fail to respond to stimulation of the medullary pyramids are also unresponsive to acetylcholine when ejected with currents up to 100 or even 150 nA, a finding which is possibly related to their very low level of spontaneous activity in anaesthetized preparations. However, even when these quiescent cells were excited by the ejection of small amounts of DLH, it

was not possible to demonstrate any excitant action of ACh upon them. It thus appears that the non-responsiveness of the cells cannot merely be due to an inadequate background depolarization (see also Krnjević and Phillis, 1963b).

At depths of 0.8 to 1.5 mm., the proportion of cholinoceptive units rises to between 12.5 per cent and 50 per cent (mean: 37 per cent) of those which could not be invaded antidromically, although the relative contribution of the 'non-Betz' cells to the entire cell population sampled at these depths is considerably smaller (see Figs. 12A and B).

In constructing these histograms, a number of 'possibly Betz' cells, and cells which were not tested for their responses to pyramidal stimulation have been included with the non-Betz group, which perhaps accounts in part for the relatively high proportion of cells responsive to ACh. However, because of the wide variations between cats in the proportion of ACh-sensitive Betz cells, any comparisons of this nature can be at best semi-quantitive.

In general, the pattern of excitation produced by ACh in these responsive but non-identified cells closely resembled that in the Betz cells in its relatively long latency, often following an early depression in the firing

rate, and with a slow offset of excitation once the ACh-ejection had ended. The doses (currents) of ACh required for excitation were also comparable.

Upon 7 cells at depths of 0.52-1.70 mm. in the visual cortex (area 17), acetylcholine was found to facilitate the firing of four cells by DLH, to have no effect on two, and possibly to depress the amino acid excitation of the remaining neurone. Again, the excitant action of acetylcholine was of slow onset and prolonged duration.

(d) <u>A comparison of responses of pericruciate neurones in anaesthetized and in cerveau isolé cats</u>

Because of the remarkedly consistent pattern of depth beneath the surface, random spontaneous activity and sensitivity to acetylcholine of the Betz cell population in cats anaesthetized with various agents, it was hoped to be able to demonstrate a similar pattern in pericruciate neurones of <u>cerveau isolé</u> preparations, although it was not possible in these latter preparations to identify the Betz cells by antidromic invasion. This comparison is summarized in Table VI and Fig. 15B. The majority of cells which exhibited spontaneous activity were cholinoceptive, and the distribution in depth of this group was very similar to that of the Betz cells within the range 0.7-1.2 mm. (Fig. 15B), but there were significantly more ACh-sensitive cells in the superficial

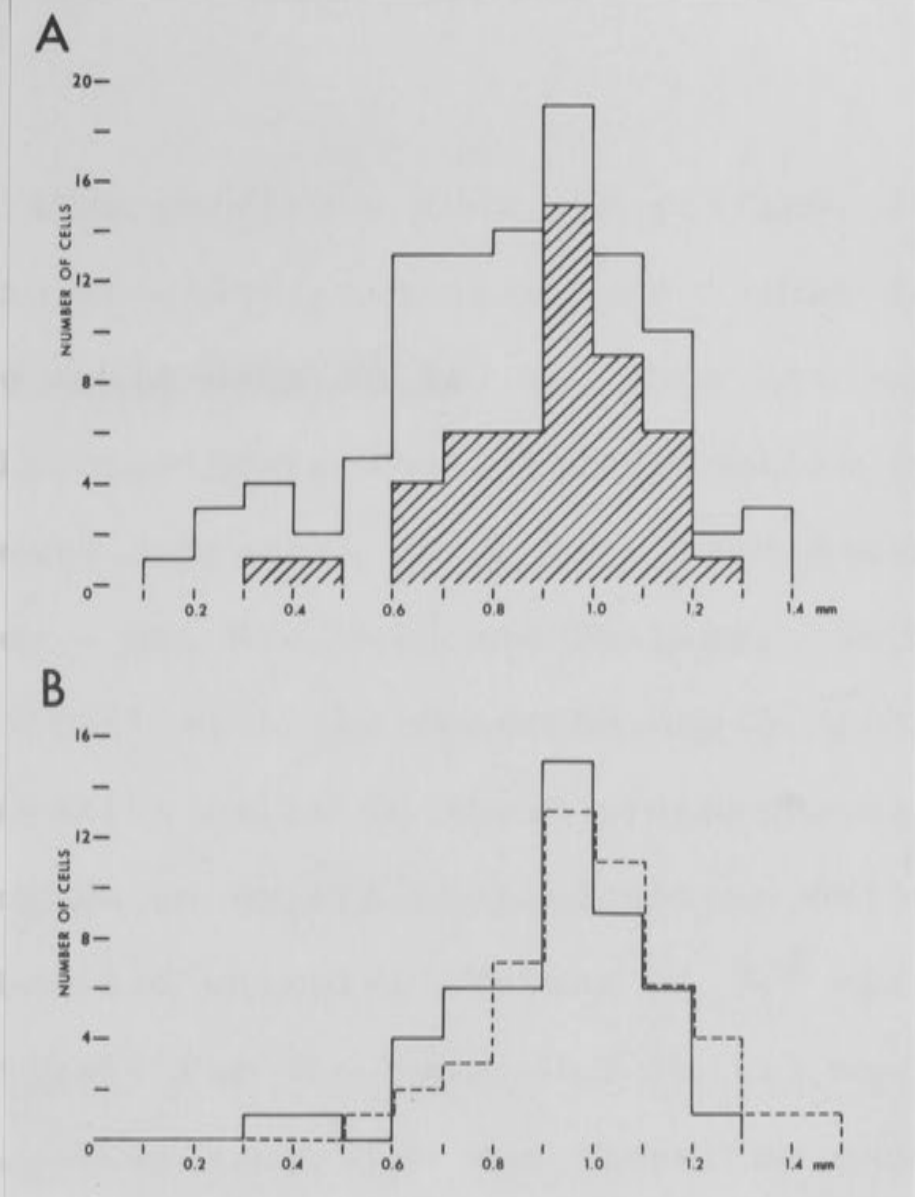

Fig. 15: A: Depth distribution of cells in the pericruciate cortex of unanaesthetized cerveau isolé cats. The acetylcholine-sensitive cells are shown hatched.

B: Depth distribution of ACh-sensitive cells in cerveau isolé preparations (solid line) compared with that calculated on the basis that these cells are members of the Betz-cell population (broken line). For clarity, the "predicted" histogram has been shifted slightly to the right.

Ordinate: Number of cells in each class interval.

Abscissa: Depth in 0.1 mm class intervals.

layers than predicted from the pattern observed with Betz cells in anaesthetized cats, and rather fewer cholinoceptive cells deep to 1.2 mm. than was expected.

The hypothesis that cholinoceptive cells in cerveau isolé cats represent Betz cells (and other deep pyramidal neurones - cf. Krnjević and Phillis, 1963b) yields a better 'fit' with the observed depth-distribution of the ACh-sensitive cells in these preparations than does a sampling based on all cholinoceptive units found in the anaesthetized animals. Values of χ^2 are 1.14 and 3.13 respectively for the range 0.7 to 1.3 mm. (in 0.1 mm. steps). The assumption was therefore made in later experiments that cholinoceptive cells between these depths in cerveau isolé preparations were most probably Betz cells. They undoubtedly resemble the pyramidal tract neurones closely in many respects, including their relative sensitivity to various cholinomimetics and acetylcholine antagonists, and their behaviour toward anaesthetic agents.

(e) Action upon hippocampal cortical neurones

A total of 19 cells in three preparations was investigated, using currents of 30-100 nA to eject acetylcholine at depths of 0.20 to 2.48 (usually less than 0.5 mm.) beneath the exposed dorsal surface of the hippocampus. Identification of the cells as pyramidal

could be accomplished on the basis of depth beneath the surface in shallow tracks (less than 0.8 mm.) made at right angles to the cortical surface, and by the prolonged pause in spontaneous or chemically-induced firing which followed stimulation of the fimbria (cf. Andersen, Eccles and Løyning, 1964a; Biscoe and Straughan, 1965).

Seven hippocampal pyramidal neurones were directly excited by acetylcholine, and four more showed facilitation of the excitant action due to DL-homocysteic acid. Excitation was usually of slow onset, with a gradual increase to a peak rate which might or might not be sustained. When the current which ejected acetylcholine was terminated, there was usually an extremely rapid increase in the firing rate of the cell, after which excitation subsided over 5-20 seconds. These features are illustrated in Fig. 16, in which the 'after-excitation' was exceptionally prominent.

Apart from the delay in onset or excitation and the rapid after-excitation, both of which would have been augmented by the onset and offset, respectively, of depression by the cationic current used to eject acetylcholine, there was no evidence of any depressant action by ACh upon these neurones.

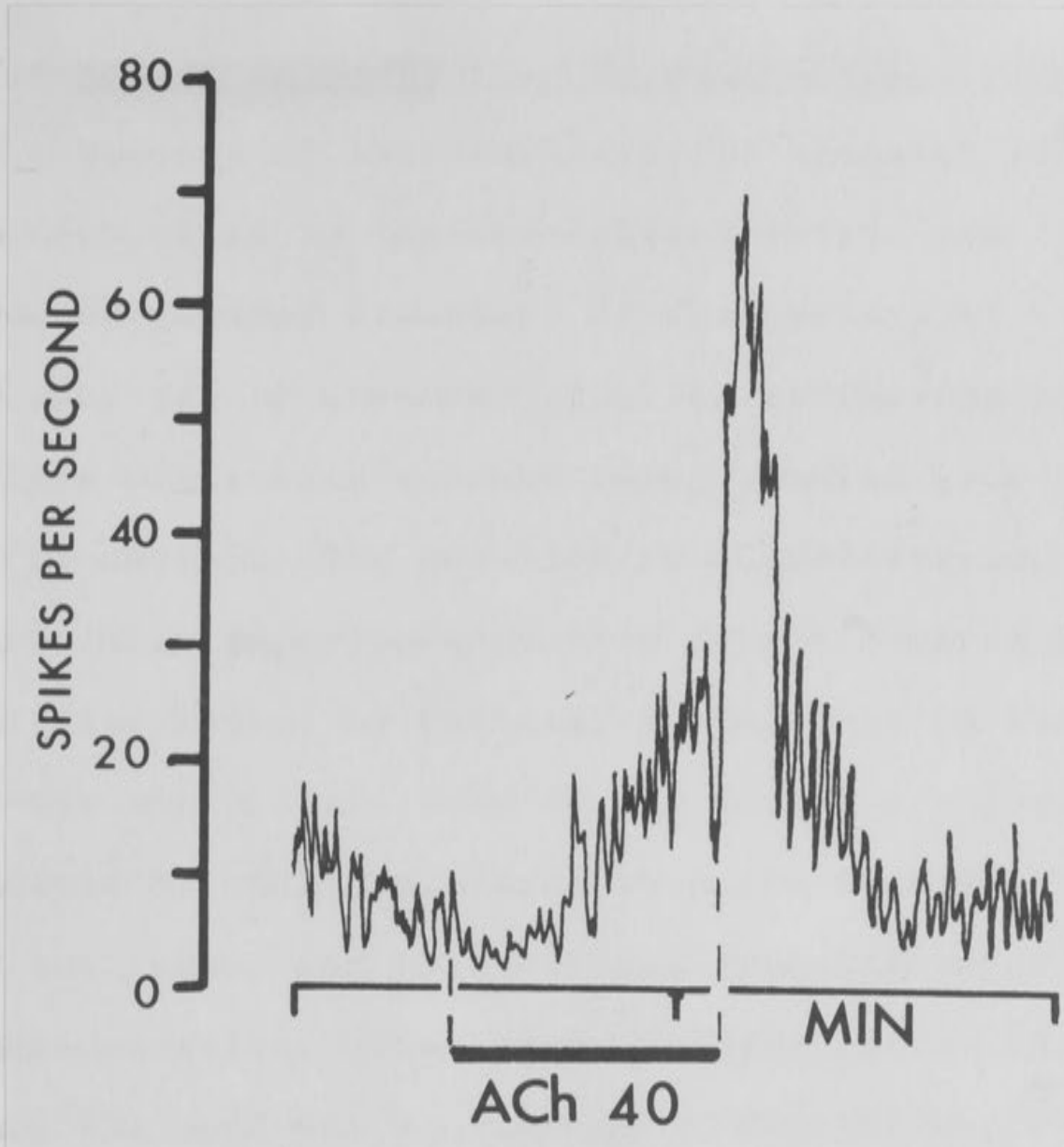

Fig. 16: Excitation of a hippocampal pyramidal cell by acetylcholine, ejected with current of 40 nA. At the termination of the ejection, there is a marked increase in firing rate, which subsides more slowly. For further description, see text.

(f) Action on cerebellar Purkinje cells (P-cells)

Because of the complexity of synaptic interconnection between cells in the cerebellar cortex, and the well-ordered laminar structure of the cortex, it was possible to make use of a number of criteria for the identification of any particular neurone being studied (see also Section II(h) above). The question of identification is of particular importance in view of the reports (McCance and Phillis, 1964a, b; Phillis, 1965a) that Purkinje cells of the superficial layer of the cortex are less readily excited by cholinomimetics than are P-cells in the depths of the folia, and that certain granular-layer cells are cholinoceptive. The latter finding raises the possibility that the apparent excitation of P-cells may be the indirect result of prior excitation of underlying granule cells by diffusion of the cholinomimetic. Accordingly, the experiments of Section VI(c) were designed in an attempt to demonstrate such trans-synaptic activation, by ejecting acidic amino acids and cholinomimetics near the presumed location of granule cells while monitoring the behaviour of a Purkinje cell with another microelectrode.

Microelectrode penetrations in the cerebellar vermis were always made perpendicularly to the surface of a folium in such a position as to avoid the convolutions of cortical

layers near the interfolial fissures, and were restricted in depth to the most superficial 0.6 mm. in virtually all experiments. The Purkinje and granule cell layers were thus approached perpendicularly and through a minimal depth of cortical tissue, and the position of the electrode tip beneath the surface could be used to indicate the position of a cell within the cortical layers. Each recording site was checked with reference to the cortical surface on the entry and withdrawal of the pipette, the observation of the surface being made under x40 magnification.

It was rare to encounter more than one neurone per track (limited in depth as outlined above) which could be identified as a P-cell. Extracellular negative-positive spike potentials were recorded at depths of 150 to 400 μm, except where tracks were made near the edge of a folium and the folding of the layers enabled the microelectrode to pass nearly parallel to the layer of P-cell bodies. Most P-cells had a 'spontaneous' firing rate of 20-80 spikes per second, some of which probably reflects damage to the dendritic tree by the microelectrode. Even so, it was usually possible to record from these cells for as much as $1\frac{1}{2}$ hours without further deterioration being observed.

Acetylcholine was ejected electrophoretically with currents of 30-120 nA near 53 identified P-cells, of which 40 (75 per cent) were excited. A further 63 unidentified cells were also tested, and of these 20 (32 per cent) were sensitive to ACh. As this latter group included cells for which only one of the above criteria was satisfied, and also cells at depths of 1-1.8 mm. beneath the surface in tracks close to a sulcus between two folia, it is highly probable that many of the group really were P-cells, but were unaffected either from that portion of the fastigial and/or inferior olivary nuclei which was stimulated, or by those basket cells which responded to surface stimulation near the crest of the folium. The inhibitory fringe due to basket cell activity extends only for a limited extent 'off-line' on either side, a distance related to the transverse spread of basket cell axons (see also Fox, 1962). It was felt unwise to attempt to re-position the concentric stimulating electrodes in the cerebellar nuclei because of possible damage to the nucleus, but occasionally this must have caused us to fail to identify cells which were in fact P-cells.

Characteristically the excitation by acetylcholine of P-cells (and these other unidentified neurones) was of slow onset when compared with that by DL-homocysteic acid, and was often preceded by a short-latency fall in the

spontaneous firing of the cell (Fig. 17). The latency of firing was quite variable, being between 5 and 30 seconds for most cells. There was then a fairly gradual increase in firing-rate, the peak value occurring after a further 10 seconds or so. At the end of the ejection of acetylcholine the rate declined to its initial level over 15-45 seconds. As with Betz cells (Section V(b) above) the initial depression of the firing rate by ejection of acetylcholine was in part due to the flow of the cationic current. However, there was always some recovery of the firing rate despite the maintenance of the current responsible for the acetylcholine ejection. In some cases this 'breakthrough' excitation by ACh remained less than the original background rate (Fig. 17B), but sometimes it would exceed this value (Fig. 17A). At the termination of current, a sudden increase in rate was observed, as seen with hippocampal pyramidal cells (compare Figs. 16 and 17) and occasionally with Betz cells also.

The passage of a cationic current through an adjacent (NaCl-containing) micropipette barrel produced a depression of the firing rate of sudden onset and offset, as a rule with associated changes in spike size, and lacking the recovery seen during the ejection of ACh (Fig. 17B). Where a P-cell was considerably depressed by a cationic current, the passage of an anionic current from the same

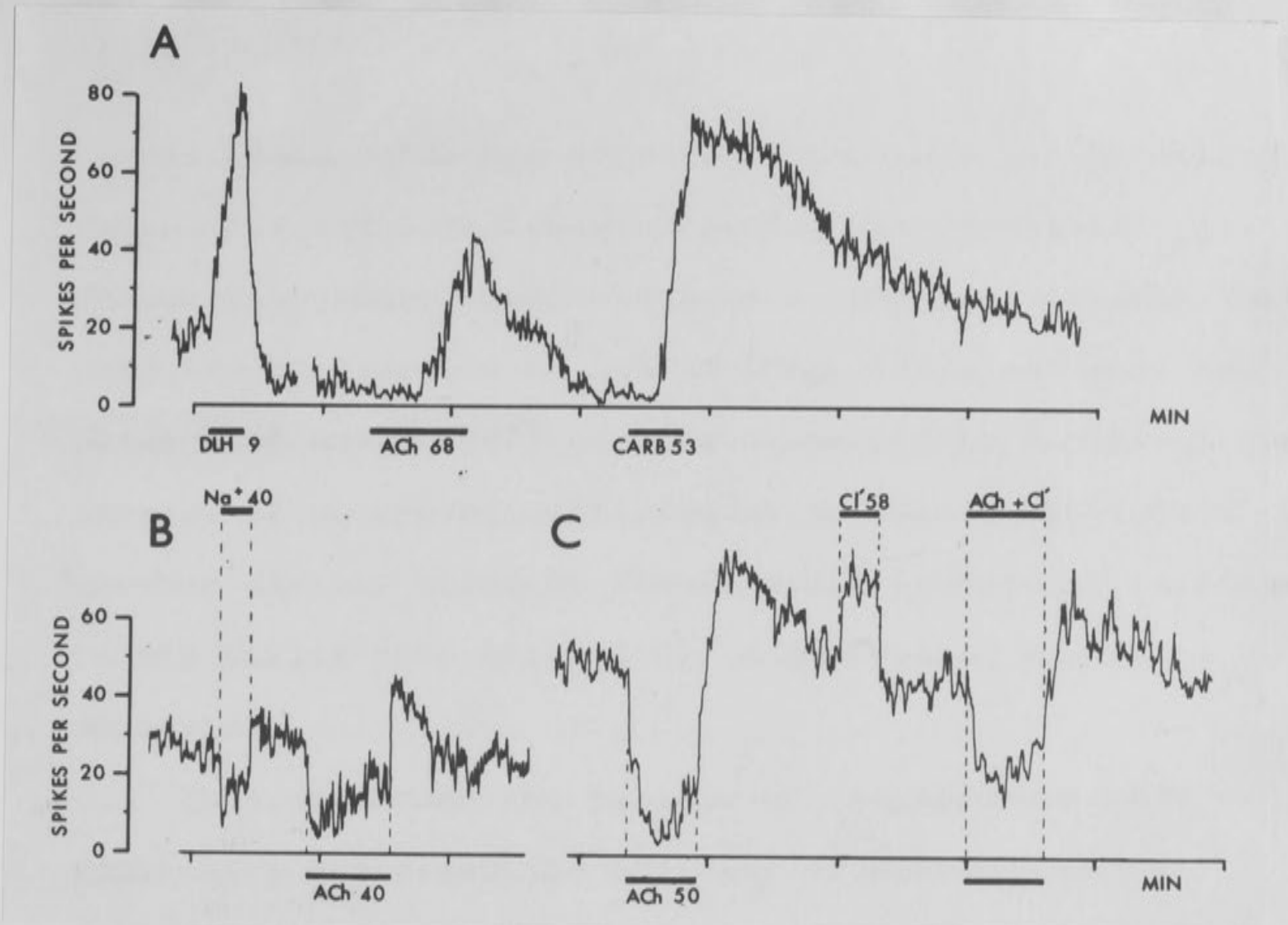

Fig. 17: Excitant and depressant effects of cholinomimetics on Purkinje cells.

A: Excitation by acetylcholine (ACh, 68 nA) and by carbaminocholine (Carb, 53 nA), compared with that by DL-homocysteic acid (DLH, 9 nA).

B: A comparison of the initial depression and after-excitation produced in another P-cell by acetylcholine (ACh, 40 nA) with the depression due to cationic current (Na^+, 40 nA).

C: Effects upon a third Purkinje cell of acetylcholine (ACh, 50 nA), an anionic current carrying chloride ions from a second micropipette barrel (Cl', 58 nA) and concurrent ejection of both ACh and the "neutralizing" current (ACh+Cl'). Further description in text.

Ordinate: Firing frequency in spikes per second.

Abscissa: Time scale in minutes.

barrel would produce a comparable increase in the firing rate. For equal cationic currents, the depression produced by acetylcholine was as a rule greater than that produced by ejection of sodium (Fig. 17B), and when the passage of acetylcholine was accompanied by the simultaneous passage of an approximately equal anionic current from another barrel, which by itself would produce an increase in the firing rate (Fig. 17C), a depression was still observed.

Thus, although the passage of cationic currents apparently depressed the activity of Purkinje cells, acetylcholine has both excitant and depressant effects. The latter is of short latency and brief duration once the ACh-administration has been terminated, and during a long ejection of ACh is apparently overcome in many instances by the more slowly developing excitation.

(g) Action on granule-layer cells of the cerebellum

Using the relatively large micropipettes of the present series of experiments, it was virtually impossible to record extracellular spike responses of single cells located at depths exceeding 350 μ. Usually, at these depths, numerous small spikes were recorded at any one position of the micropipette, but it proved very difficult to identify any of these with granule cells. The

'spontaneous' discharge rate of these groups was high, and could be further increased by the ejection of small doses of DL-homocysteic acid, but in a number of experiments with both acetylcholine and carbaminocholine, using electrophoretic currents of up to 120 nA for periods as long as one minute, there was no apparent excitation of these groups of granule-layer cells.

It has thus not proved possible to substantiate reports of excitation of cells in the granular layer of the cerebellum (McCance and Phillis, 1964a, b, 1965). However, caution must be exercised in accepting the identification of cell types on the basis of histological lesioning techniques. McCance and Phillis recorded the summed activity of groups of cells in the granular layer, using even larger (8-12μ diameter) micropipettes than those of the present series of experiments, and report a 'tendency for the amplitude of granule cell layer activity to increase as the electrode approached a Purkinje cell layer' (McCance and Phillis, 1964b). The lesions made by the passage of hydrogen ion are fairly large (100-200μ ; McCance and Phillis, 1964b, 1965), and although the centre of such a lesion indicates the location of the microelectrode, it is conceivable that units at comparatively great distances from the electrode tip may have been amongst those recorded, especially in view of the damage

caused to the cells nearest the electrode. In our experiments (Section II(e) above), it was usually possible by careful movement of the electrode to isolate a single acetylcholine-sensitive neurone with a large (greater than 200 μV) spike from amongst any such 'groups' as were apparently excited by ACh. This single cell could then in virtually all cases be identified as a Purkinje cell, either by the occurrence of spontaneous 'inactivation responses', or by antidromic invasion after stimulation of the ipsilateral fastigial nucleus. Thus, ACh-sensitive neurones apparently located in the granular layer may in fact have been Purkinje cells at some distance from the micropipette tip (Crawford and Curtis, 1965).

(h) Summary

Whereas a large proportion of Betz, hippocampal pyramidal and Purkinje cells are excited by electrophoretic administration of acetylcholine, not all members of these populations have been found to be responsive, even in unanaesthetized preparations. Conversely, some 'unidentified' (possibly insufficiently identified) cells do respond to ACh ejection by a similar gradual excitation with slow offset as do these large cells of the respective cortical efferent pathways.

A full discussion of the significance of the excitation by ACh in relation to the synaptic firing of the cells will be found in Sections VIII and XI, after the results of further pharmacological analysis have been presented. In general, however, it appears that the long time-courses of excitation of various types of cortical neurone by acetylcholine are quite different from those of Renshaw cells (Curtis and Eccles, 1958; cf. however, Curtis and Ryall, 1965a). Depressant actions of ACh are also relatively prominent on the cortical neurones, and probably contribute to the initial depression even of cells which are eventually excited. The dramatic increase in firing rate which followed the end of the administration of ACh (e.g. on the hippocampus, Fig. 16, or cerebellum, Fig. 17B) probably indicates a short duration of action of ACh-depression, but detailed studies have not been undertaken.

Although cerebellar Purkinje cells were readily excited by acetylcholine, caution is necessary in accepting the reports of ACh-sensitive cells of the granule cell layer (McCance and Phillis, 1964) even though these latter findings would be in keeping with the histochemical demonstration of acetylcholinesterase in mossy fibre afferent systems to the granular layer (reported in Mead and Van der Loos, 1964; Austin, Phillis and Steele, 1964;

Friede and Fleming, 1964; Shute and Lewis, 1964, 1965; Phillis, 1965a, b). These findings are further discussed in Section XI(b).

SECTION VI - OTHER CHOLINOMIMETIC AGENTS AND ANTICHOLINESTERASES

(a) Studies of cerebral cortical neurones

The results to be presented broadly confirm the generalization (Krnjević, 1964) that many choline esters are quite effective excitants of cortical neurones, usually in proportion to their muscarinic potency. It has also been found that the time-courses and relative potencies of various cholinomimetics are identical whether assessed on identified Betz cells or upon those cells in unanaesthetized cerveau isolé preparations which correspond in depth, 'random' spontaneous activity and sensitivity to ACh itself to the Betz cell population (see also Section V(d) above). This latter result lends support to the hypothesis that the populations are in fact similar, as it was assumed in a later series of experiments on anaesthetic actions (Section X).

As with the estimation of the relative potencies of amino acids (Section III(b)), care was always taken to continue each drug ejection for a sufficiently long time as to ensure that maximal effects for the dose were being observed. The comparisons were made upon several cells in different cats, wherever possible repeating the estimation at various levels of evoked firing frequency.

In this manner the effects of differences in the time-course of excitation upon the estimate of potency are minimised, but it must be again emphasised that these comparisons are at best semi-quantitive.

Because of difficulties associated with their interpretation, relatively few experiments have been performed using electrophoretic or systemic administration of anticholinesterases, and it is convenient to include these experimental results also in this Section. When ejected near Renshaw cells (Curtis and Eccles, 1958b; Curtis, Phillis and Watkins, 1961; Curtis and Ryall, 1965), anticholinesterases frequently show a direct excitation which might mask any prolongation or accentuation of ACh sensitivity or synaptic firing. On the other hand, intravenous injection of anticholinesterases produces marked systemic effects (muscular twitching, changes in heart rate and blood pressure, salivation, etc.) and could possibly produce undesired indirect effects upon the single neurone studied.

For convenience, a summary of the results presented in this Section is appended as Table VII.

(i) Carbaminocholine. In contrast to the findings of Krnjević and Phillis (1961b, 1963c), where carbaminocholine was reported to have only a weak excitant action

upon Betz cells, no consistent difference was found between the potencies of carbaminocholine and ACh. A total of 51 direct comparisons was made upon Betz and unidentified cortical neurones in both anaesthetized and unanaesthetized cerveau isolé preparations. The ratio of the currents which ejected equi-effective concentrations of the choline esters was found to vary within the range 0.7 to 1.3 (carbachol: ACh), but the onset of firing with carbaminocholine was often slower and the decay almost always more prolonged than that seen with acetylcholine (cf. also Krnjević, 1964, page 73).

In the case of the Betz cell (located at 0.99 mm. depth) illustrated in Fig. 18, acetylcholine ejected with a current of 80 nA produced a firing rate of some 40-45 spikes/second, as did carbaminocholine ejected with a current of 62 nA. The potency ratio here was thus 1.3, and the offset after carbaminocholine was some $2\frac{1}{2}$ times as long as after ACh. Essentially similar results upon a non-pyramidal tract neurone are shown in Fig. 19, where approximately the same rate of firing was produced by ejection of the same dose (current = 80 nA, through molar solutions) of ACh, carbaminocholine and acetyl-β-methyl-choline. Between each cholinomimetic substance the response of the cell to DL-homocysteic acid was checked,

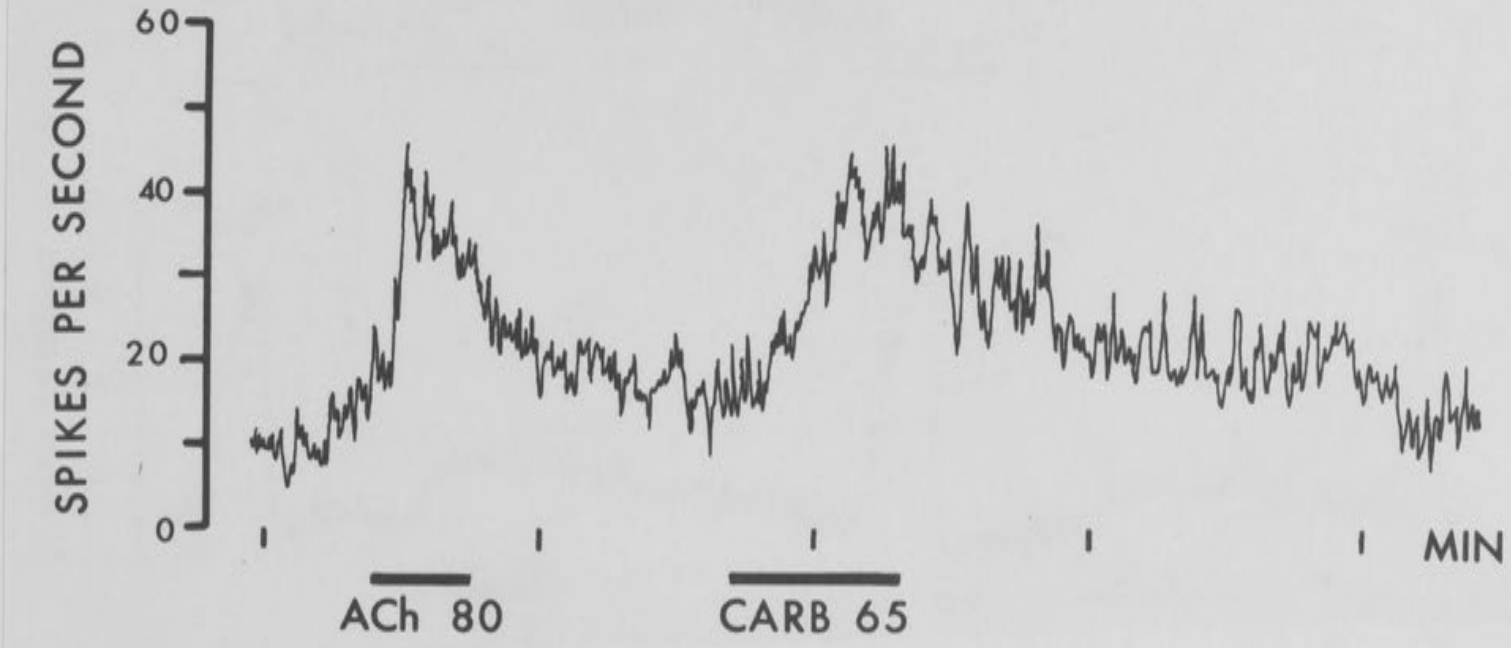

Fig. 18: Firing frequency of a Betz cell, excited by administration of acetylcholine (ACh, current of 80 nA) and carbaminocholine (CARB, 65 nA). The difference in decay-times of the excitation by these agents is less marked than in ~~the~~ other cells.
Ordinate: Firing frequency (spikes/second).
Time scales in minutes.

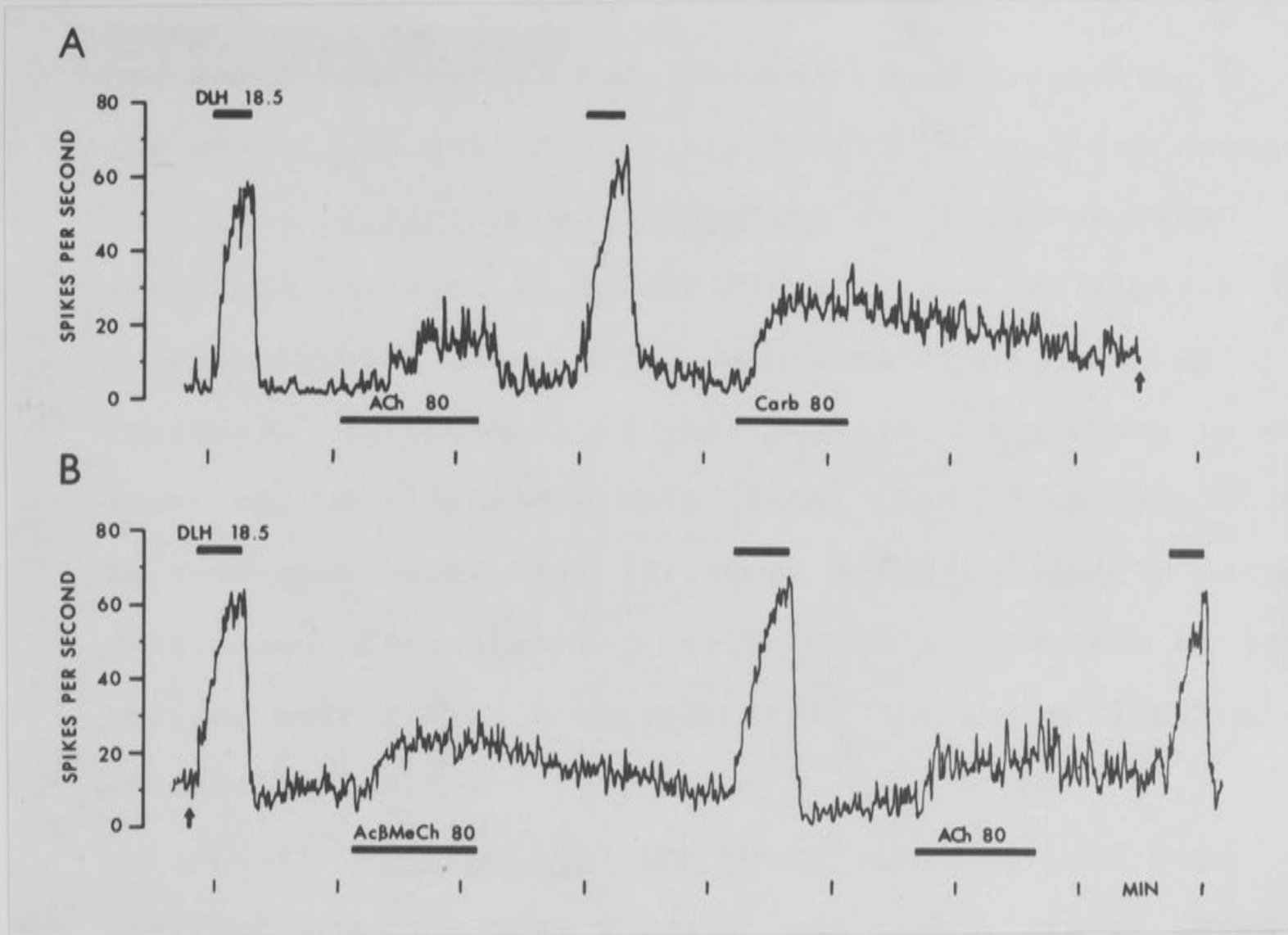

Fig. 19: A comparison of various cholinomimetics upon an unidentified pericruciate neurone. Equal currents (80 nA) were used to eject acetylcholine (ACh), carbaminocholine (CARB) and acetyl-β-methylcholine (AcβMeCh), and the sensitivity of the cell to DL-homocysteic acid checked between each drug administration. The traces A and B are continuous at the point marked by the arrows.

Ordinate: Firing frequency in spikes/second.

Time scales in minutes.

and the difference in time parameters of excitation by the amino acid and the choline esters is well demonstrated.

(ii) Acetyl-β-methylcholine was compared with acetylcholine upon 13 cells, yielding closely similar results on both Betz cells and unidentified cortical neurones. The potency of this substance was found to be equal to, or slightly greater than, that of acetylcholine in each case (e.g. Fig. 19, lower trace). The offset of excitation after acetyl-β-methylcholine was also prolonged, lasting half a minute or more after the end of the drug administration.

(iii) Propionylcholine (PrCh) also excited the cortical neurones upon which it was tried, all of which had previously been found to be sensitive to acetylcholine. This compound was considerably less active than acetylcholine upon both Betz and unidentified cells. In the example shown as Fig. 20, 30 nA of propionylcholine fired a Betz cell less effectively than did 10 nA of acetylcholine, although 60 nA of propionylcholine was rather more effective than the ACh. In this case, therefore, the potency of PrCh would be assessed as 0.3. The firing produced by propionylcholine was usually more prolonged than that of ACh, although this feature is not demonstrated in Fig. 20.

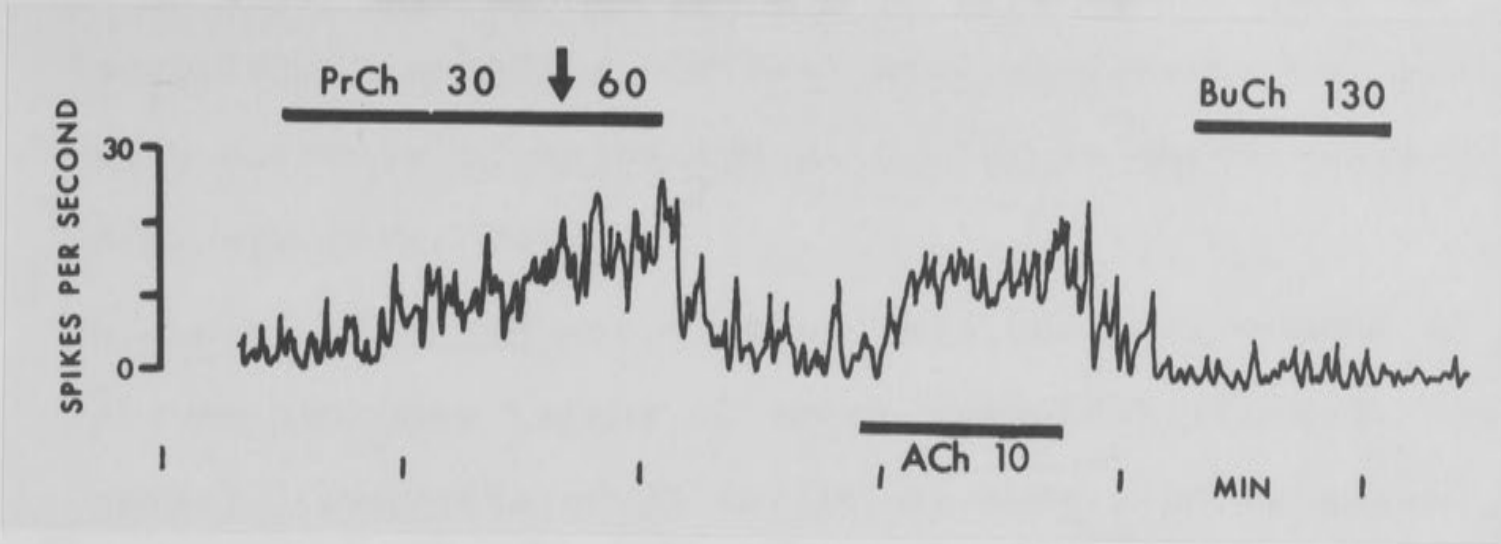

Fig. 20: Comparison of effects of propionylcholine (PrCh), and n-butyrylcholine (BuCh) with those of acetylcholine upon a Betz cell. The currents (in nA) used for each ejection are shown beside the appropriate signal bar.
Ordinate: Firing frequency (spikes/second).
Time scale in minutes.

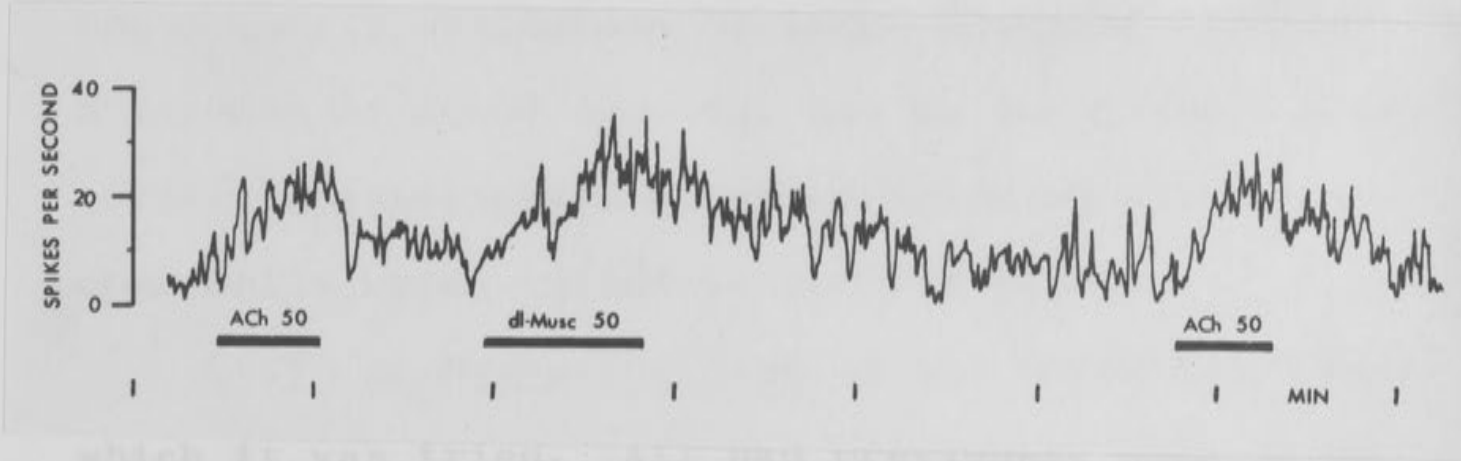

Fig. 21: Actions of acetylcholine (ACh, current of 50 nA) and dl-muscarine (dl-Musc, 50 nA) upon another Betz cell.
Ordinate: firing frequency in spikes/second.
Time scale in minutes.

(iv) n-Butyrylcholine did not appear to excite acetylcholinoceptive cortical neurones, even when ejected with currents of up to 130 nA for up to three-quarters of a minute (Fig. 20).

(v) Nicotine was ejected into the environment of eleven neurones (eight of which were identified as Betz cells). Currents of 75 to 100 nA were used to eject nicotine from molar solutions. Five of the cells (three of which were Betz cells) were excited, but the potency of nicotine was only one-half to three-quarters that of acetylcholine upon the same cells. Excitation by nicotine was often irregular and accompanied by much extraneous activity in neighbouring cells, but the latency of onset was not significantly longer than with ACh. Krnjević and Phillis (1963a, c) have described a non-specific excitation by large doses of nicotine, with a latency of 10-40 seconds, but in the present short series of experiments nicotine appeared to have no effect upon cells which failed to respond to ACh.

(vi) dl-Muscarine excited all seven cells upon which it was tried. All had previously been shown to respond to acetylcholine, and the potency of muscarine on these neurones was found to vary from 1-4 times that of ACh. The offset of firing after dl-muscarine

was usually longer than that which followd ACh-ejection (Fig. 21), and on one occasion firing persisted for over five minutes after muscarine had been ejected with a current of 80 nA. This appears to be an extreme example of the tendency of Betz cells to fire for prolonged periods after chemical excitation with many of the cholinomimetics.

(vii) Oxo-tremorine (1-(2-oxopyrrolidino)-4-pyrrolidino-butyne-2) is a muscarinic agent equal in potency to acetylcholine but lacking the nicotinic activity of the latter (Cho, Haslett and Jenden, 1962). When ejected upon three cells in a cerveau isolé preparation, all of which had been shown to be excited by acetylcholine, two were fired by comparable doses of oxo-tremorine. Upon noncholinoceptive cells in the same preparation it had no apparent effect.

(b) Studies upon cerebellar Purkinje cells

All experiments were performed on cats anaesthetized with pentobarbital sodium (35-40 mg./kg.), and the methods used to identify these neurones were as outlined in Section II(h)ii above.

(i) Carbaminocholine excited 60 of the 68 identified Purkinje cells upon which it was ejected, and was also effective in 39 out of 56 unidentified neurones (some of which may have been P-cells - cf. Section II(g)ii above). Whenever ACh and carbaminocholine were compared upon the same cell the latter was found to be more potent than ACh, and to have a more persistent action after the ejection had ceased. Following the cessation of ejection, durations of action of up to three minutes were frequently seen, and occasionally the background rate remained consistently higher than the control value for prolonged periods.

(ii) Acetyl-β-methylcholine was similar in potency to acetylcholine in comparisons made on nine cerebellar neurones in three cats, but in all cases the recovery after this compound was much slower than that after ACh.

(iii) Nicotine was also comparable in potency with acetylcholine when ejected upon P-cells, but was considerably less effective than carbaminocholine. Its duration of action was intermediate between those of the choline esters, usually persisting for 30-45 seconds after the end of the ejection.

(iv) dl-Muscarine was tested on only three P-cells, but proved to be as potent as carbaminocholine as an

excitant of these cells, and again had a more persistent action than ACh.

(c) Actions upon cells in the cerebellar granule-cell layer

As mentioned previously, it was not possible to select a single cell from amongst the numerous small spikes recorded when the microelectrode was lowered to depths of 0.4 to 0.7 mm. from the surface. Accordingly, a less well-defined method of assessing activity had to be used, and attempts were made to alter the overall spike activity of all neurones whose spikes as recorded by the micro-electrode at a given position were greater than about 100 V. Such a test allows only qualitative conclusions to be drawn as to the efficacy of an excitant upon the masses or groups of cells being recorded.

The high 'spontaneous' discharge rate of these groups was attributed in part to damage by the micropipette, but although the ejection of DL-homocysteic acid caused a further increase in the firing rate, neither acetylcholine nor carbaminocholine (with currents of up to 120 nA for one minute) nor dl-muscarine (currents of 60 nA) produced any evidence of excitation of these cells.

Two-electrode experiments

Assuming the granule cells to be chemically excitable, it was anticipated that ejection of appropriate excitants

into the granule cell layer (0.4 to 0.6 mm. depth) would cause Purkinje cells 'in line' with the ejection site to be fired synaptically, as the parallel fibres (granule cell axons) run in the axis of the folium. In Fig. 4, for instance, ejection from electrode 'A' would be expected to fire the P-cell near electrode 'B'. On the other hand, an 'off line' Purkinje cell, 100-500μ transversely from the folial axis through the point of ejection (such as that recorded by electrode 'C' in Fig. 4) would not be itself excited from the activated portion of the parallel fibre system, but would be subject to post-synaptic inhibition by the basket and stellate cells whose dendritic trees lie 'in line', but whose axons spread transversely to the P-cell under observation. This inhibitory action on 'off line' Purkinje cells could be demonstrated as a depression of either the spontaneous or the amino acid evoked firing of the cell.

One possible source of complication in these two-electrode experiments has been the occurrence of direct effects upon the distant P-cell by diffusion of the excitant from its site of ejection. This could readily be demonstrated under circumstances which minimized the possibility of synaptic interaction, as when both the ejection and recording sites were inthe region of P-cells,

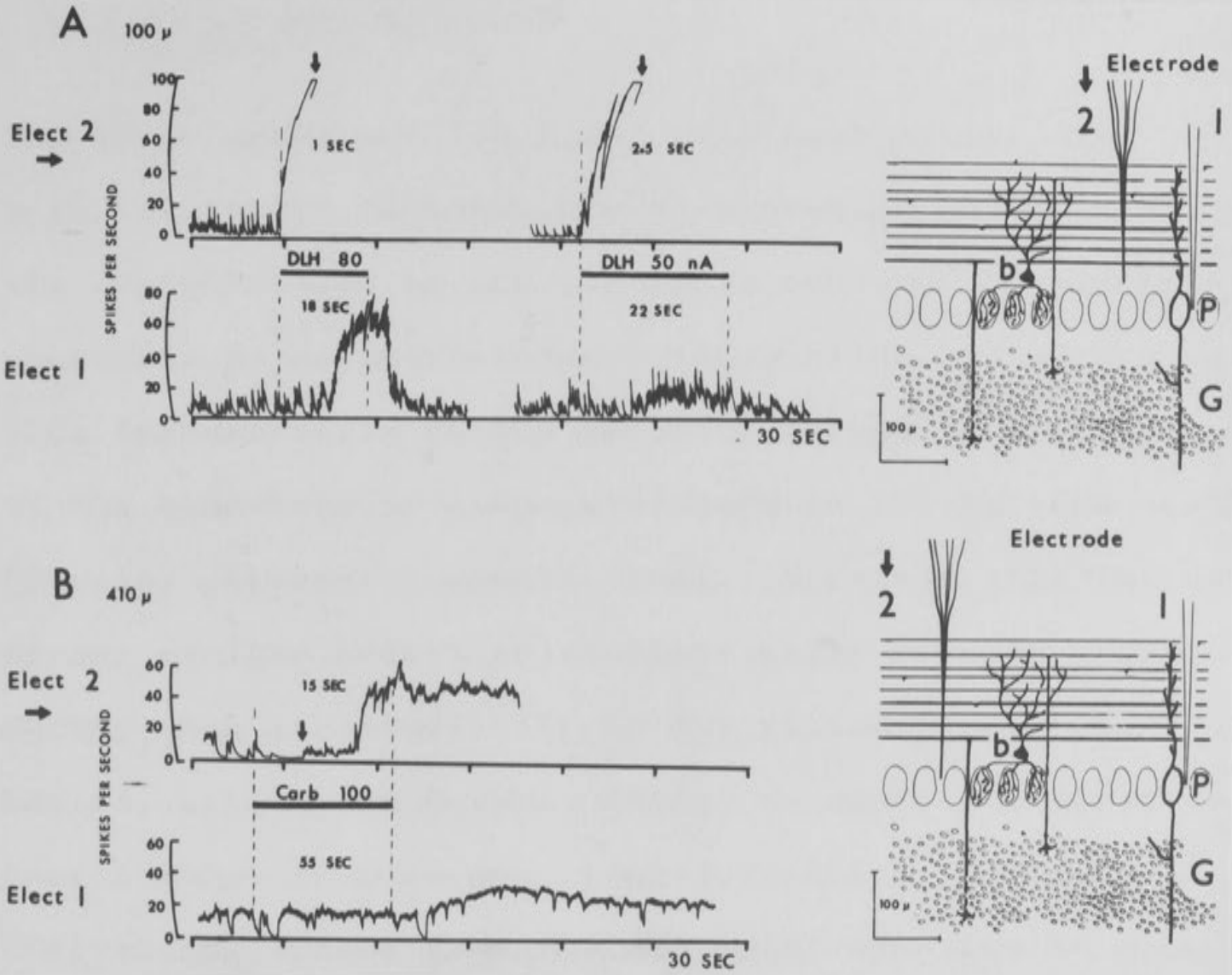

Fig. 22: "Two-electrode"experiment in a cerebellar folium, showing effects of diffusion over distances in the molecular layer. Both the ejecting ("2") and recording electrodes ("1") were close to Purkinje cell bodies, but were separated by interelectrode distances of 100μ in A, and 410μ in B. Various amounts of DL-homocysteic acid (DLH) and carbaminocholine (Carb) were found to affect the more distant cell (lower frequency traces) later than the cells near the ejecting electrode (upper traces). The latency of excitation is indicated on each trace, and the experimental arrangement is shown schematically for each pair of records. Further description in text.

Ordinates: Firing frequency (curvilinear scales)

Time scales: 30 sec. for each record.

100-400μ apart and 'in line' with each other. The parallel fibres themselves are apparently insensitive to the concentration of the excitants achieved by electrophoresis, as no short-latency interaction was ever seen. This insensitivity of the parallel fibres is similar to the behaviour of axons elsewhere in the nervous system (Curtis, personal communication). Moreover, the influence of any excited basket or stellate cells spreads transversely rather than longitudinally in the folium (e.g. Fox, 1962; Eccles, Llinas and Sasaki, 1965g) so would not affect a P-cell 100μ or more away longitudinally. Although diffusional spread from the region of ejection to granule cells below this site remains a possible means of producing synaptic interference with the distant cell in such experiments, no short latency effects of drug ejection were noted, whence this mechanism also is probably unimportant under the conditions used. Consequently the effects observed in trials such as those of Fig. 22A and B have been attributed to direct diffusion from the ejecting microelectrode (called '2' in the next five figures) to the distant P-cell being recorded by electrode '1'.

In Fig. 22A, ejection of large doses (80 and 50 nA) of DL-homocysteic acid from electrode 2 caused short-latency excitation of the P-cell near its tip, and rapid depolarization block of its spikes. This is shown on the

upper frequency trace of Fig. 22A. The P-cell near electrode 1, at a depth of 0.21 mm. and 100μ away from the point of ejection, was also fired, but with considerably longer latency and at lower frequency than the nearer cell (a much lower frequency in the case of the smaller ejection of DLH). The latency difference (17 and 19½ seconds) yields an approximate value of the time required for an adequate concentration of DLH to diffuse across to the distant P-cell. Here, the rate of movement of the drug front was some 5-6μ /second.

A similar experiment, but with the ejection of carbachol with a current of 100 nA at a point 410μ from the P-cell recorded by electrode 1, is shown in Fig. 22B. Here, the Purkinje cell near the point of ejection had a latency of some 15 seconds before firing began, and a rather atypical response to the excitant, with an abrupt increase in firing rate some 20 seconds after the start of the excitation. The distant Purkinje cell recorded by electrode 1 (lower frequency trace) had a spontaneous firing rate of some 15 spikes per second, with irregular decreases in rate occurring spontaneously. These continued during and after the ejection of carbaminocholine, but excitation of this cell by the drug was delayed until some 55 seconds after the commencement of ejection and was of gradual onset and offset. From the latency

difference between the 'neighbouring' and 'distant' P-cells, it appears that the carbachol was diffusing through the cortical tissue at some 10μ/second.

The effect upon the firing of a 'distant' P-cell of drug ejection at various depths in the cerebellar cortex are shown in Fig. 23. The recording and ejecting micro-electrodes were again 'in line', and separated by a distance of 330μ along the axis of the folium. Ejection of DL-homocysteic acid at depths of 280μ (a; at the approximate level of the Purkinje cells) or 480μ (c; in the white matter beneath the granule cell layer of this folium) produced no change in the firing rate of the P-cell recorded by electrode 1. However, ejection at depths of 380μ (b) and 400μ (d) caused excitation of the local group of granule cells (not illustrated) and short-latency unsustained excitation of the distant cell. The latency was much less than that required for diffusion across 330μ, being only some two seconds in each case, and indicates a synaptic activation of the P-cell by the excited granule cells. The fact that the P-cell excitation was not sustained is due to two factors - the rapid occurrence of depolarization block of the granule cells, and the lateral spread of DLH to excite other granule cells whose axons (parallel fibres) are 'off-line' for

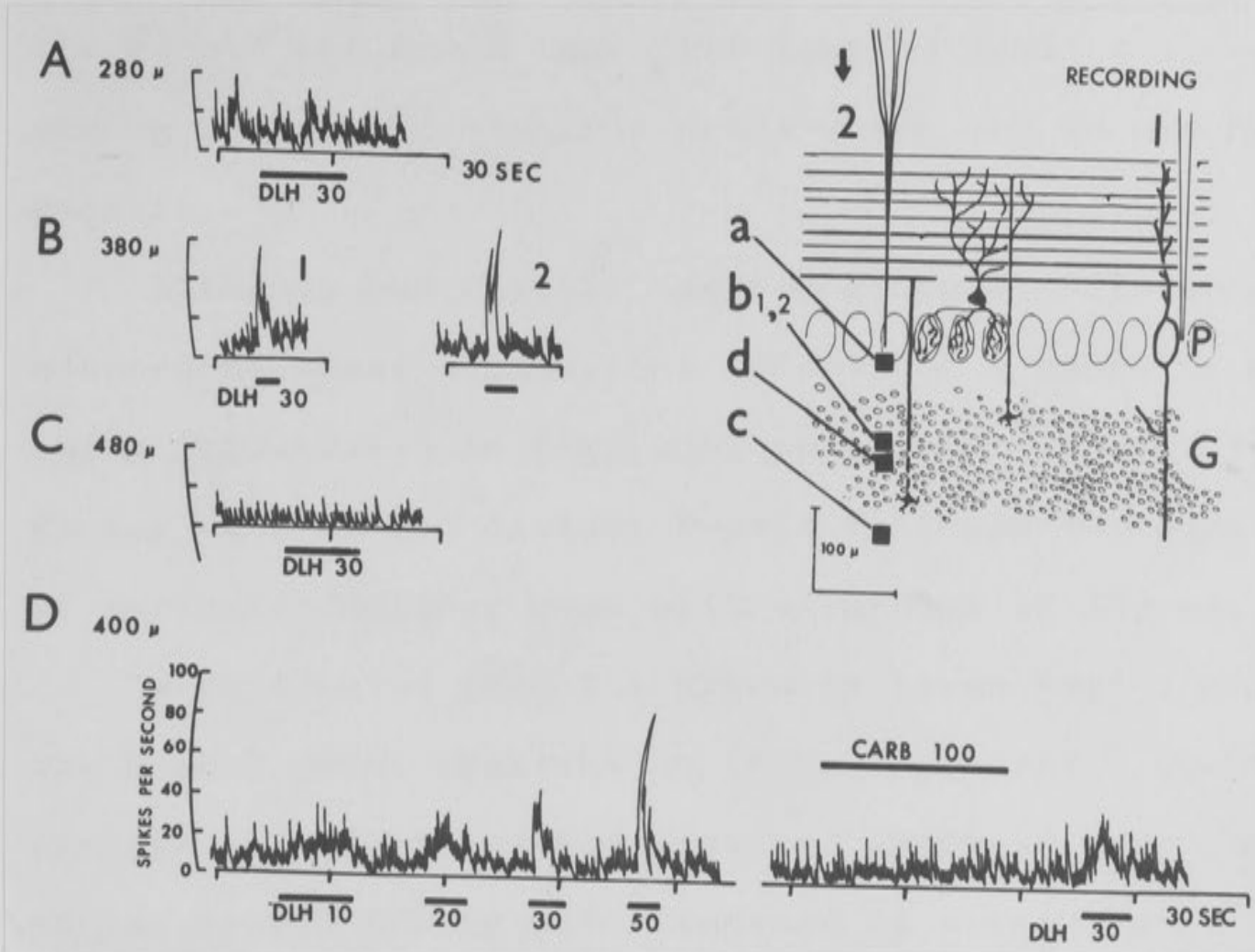

Fig. 23: Effects of ejection of DL-homocysteic acid (DLH) at various depths within the cerebellar cortex. The ejecting electrode ("2") was 330μ from the recording site (electrode "1"), and "in line" with it along the folial axis. All records of firing frequency were made by electrode "1", but the short horizontal bars beneath each trace mark the period of drug ejection from electrode "2". The experimental arrangement is indicated in the inset diagram, with the points of ejection marked by black squares. These points (a-d) correspond to the frequency traces (A-D) respectively, and the actual depth of each ejection site is shown beside the appropriate frequency trace. For further description, see text.
Ordinate: Firing frequency of Purkinje cell near electrode 1 (curvilinear scales). Time scale in 30 sec. (all traces).

the P-cell and would therefore tend to inhibit it by firing basket and stellate cells which end on the distant P-cell.

Although DLH readily excited granule cells when ejected at these depths, the effects of a range of doses being illustrated in Fig. 23D, no apparent change in the firing rate of the distant P-cell followed the ejection of carbaminocholine, even with a current of 100 nA.

When ejected into the granular layer 'off-line' for the P-cell under observation (Fig. 24, inset), DL-homocysteic acid produced the expected short-latency depression of the P-cell firing rate recorded by electrode 1. Carbaminocholine, however, had an entirely different action. After a long latency, a very gradual increase in firing rate was observed. This continued for about a minute after the end of the drug ejection and then declined gradually. The irregular brief depressions of the firing rate seen during and after the ejection of carbaminocholine in Fig. 24 were occurring spontaneously in the control period also, and were unrelated to the drug action. The slow excitation of the distant cell by carbachol was attributed to diffusion of the drug and its direct action on the P-cell being studied - again, there was no evidence of synaptic effects implying an excitant action of the cholinomimetic on granule cells.

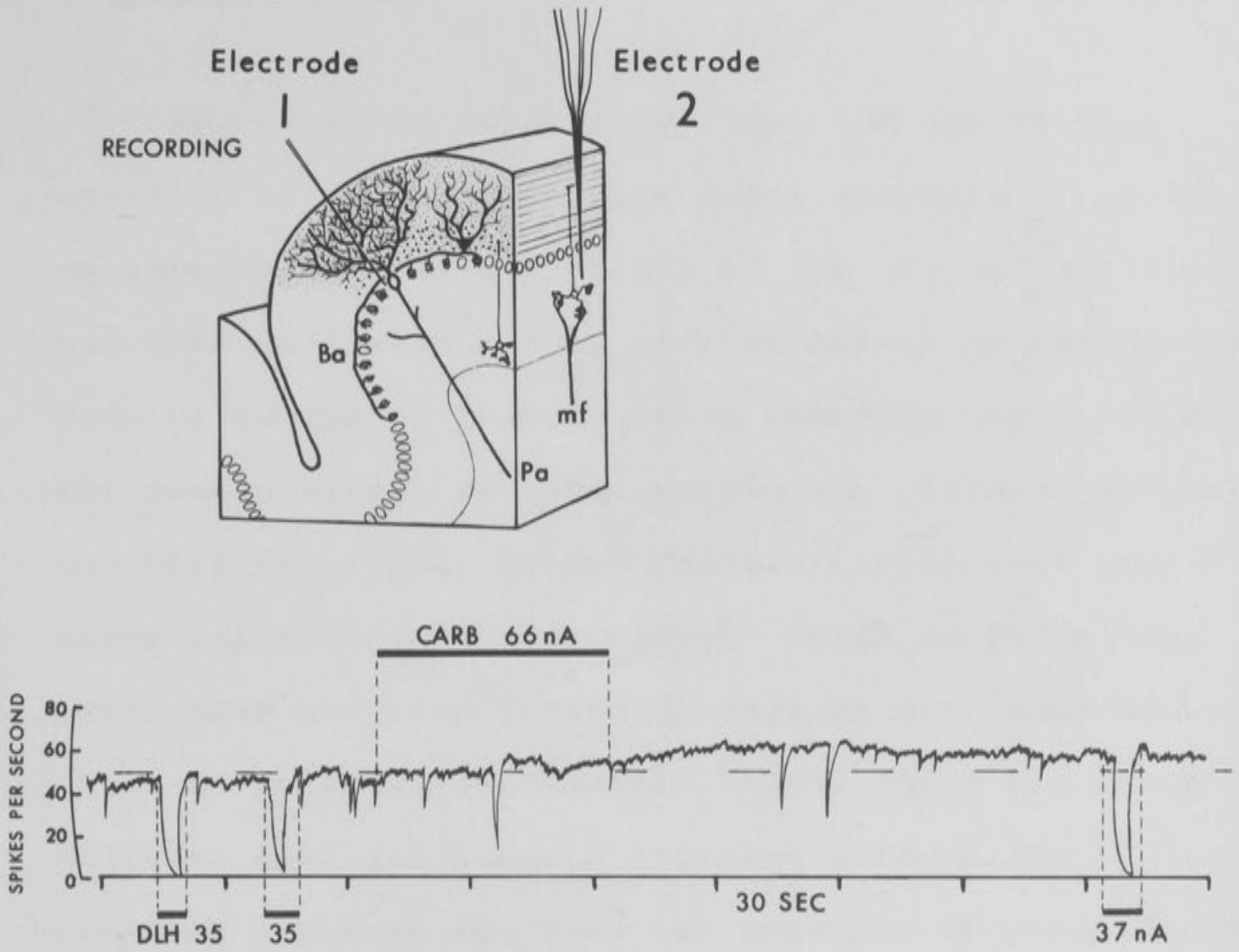

Fig. 24: Effect of DL-homocysteic acid and carbaminocholine ejected into the granule-cell layer (300μ depth) 150μ "off-line" for the distant cell. The experimental arrangement is as shown in the diagram above the frequency trace of Purkinje-cell activity recorded by electrode "1". During the periods shown by horizontal bars, drug ejections were made from electrode "2". The interrupted horizontal line at 50/second serves merely as a reference level.
Ordinate: Firing frequency of the Purkinje cell (curvi-linear scale).
Abscissa: Time scale (30 sec. intervals).

The negative results of Figs. 23D and 24 with ejection of carbaminocholine using currents of up to 100 nA were typical of all trials in the present series. As had been indicated by the lack of effect on the discharge rate of groups of granule cells (Section V(g)), it has not been possible to substantiate the claim of McCance and Phillis (1964a, b) and Phillis (1965) that granular-layer cells are cholinoceptive. Golgi cells of this layer have not been tested in this manner, however, and it is of interest that Koelle (1954, 1963) has shown moderate AChE-staining of isolated large cells of the granular layer of the rat, the majority of granule cells exhibiting no staining (see also Section XI).

(d) Experiments upon cerebellar basket cells

Attempts were made using two multibarrelled micro-pipettes inserted transversely across the folium one from another to study basket cell - Purkinje cell interaction directly, without the intervention of granule cell activity. The cell bodies of the basket cells lie deep in the molecular layer, just above those of the P-cells (e.g. Ramon y Cajal, 1911; Fox, 1962), and their axons spread transversely to enmesh the Purkinje cell bodies (Fig. 25, upper, from Szentagothai, 1963). However, it has not been possible in the present series of experiments

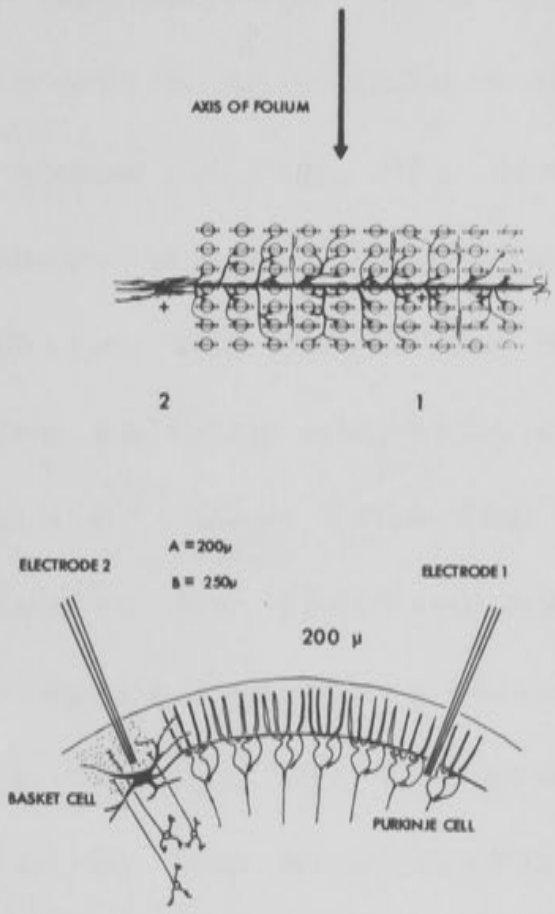

Fig. 25: Experimental arrangement used to show basket-cell interaction upon Purkinje cells (after Szentagothai, 1963). Above, schematic vertical view of cerebellar folium, to show predominantly transverse spread of basket cell axons from cell body at left. (Ejection site near this cell marked by cross above "2", recording site near P-cells by cross above "1"). Below, schematic transverse section through folium in plane of basket cell and its axon to show microelectrode placement. The two depths (200 and 250μ) refer to parts A and B of the succeeding Figure.

to identify basket cells physiologically from their extracellular spike activity (cf. Eccles, Llinas and Sasaki, 1965g), but only by their effect on the firing of a P-cell with which they make contact. The experimental arrangement is shown in Fig. 25, lower. One electrode was kept stationary and recorded the responses of a nearby P-cell whilst the other was advanced cautiously and the responses to drug ejection checked from time to time. At a depth of 200μ from the surface there were no cells near enough to the ejecting electrode ('2') to have distinguishable spike responses which could be counted (Fig. 26A, upper trace), but the presence of basket cells nearby was proved by the short-latency depression of the firing of the P-cell near electrode 1 which followed ejection of DLH, but not carbachol, from 2. As electrode 2 was then advanced some 10μ, a cell was suddenly impaled and fired extremely rapidly for almost 15 seconds before deteriorating and being eventually lost. Simultaneously with the impalment of this cell, the Purkinje cell near the other electrode was completely inhibited for some ten seconds, after which gradual recovery of its spontaneous firing was observed (Fig. 26A, end of trace).

Another comparison of the effects of DLH and carbachol ejection at a depth of 250μ near a basket cell is shown

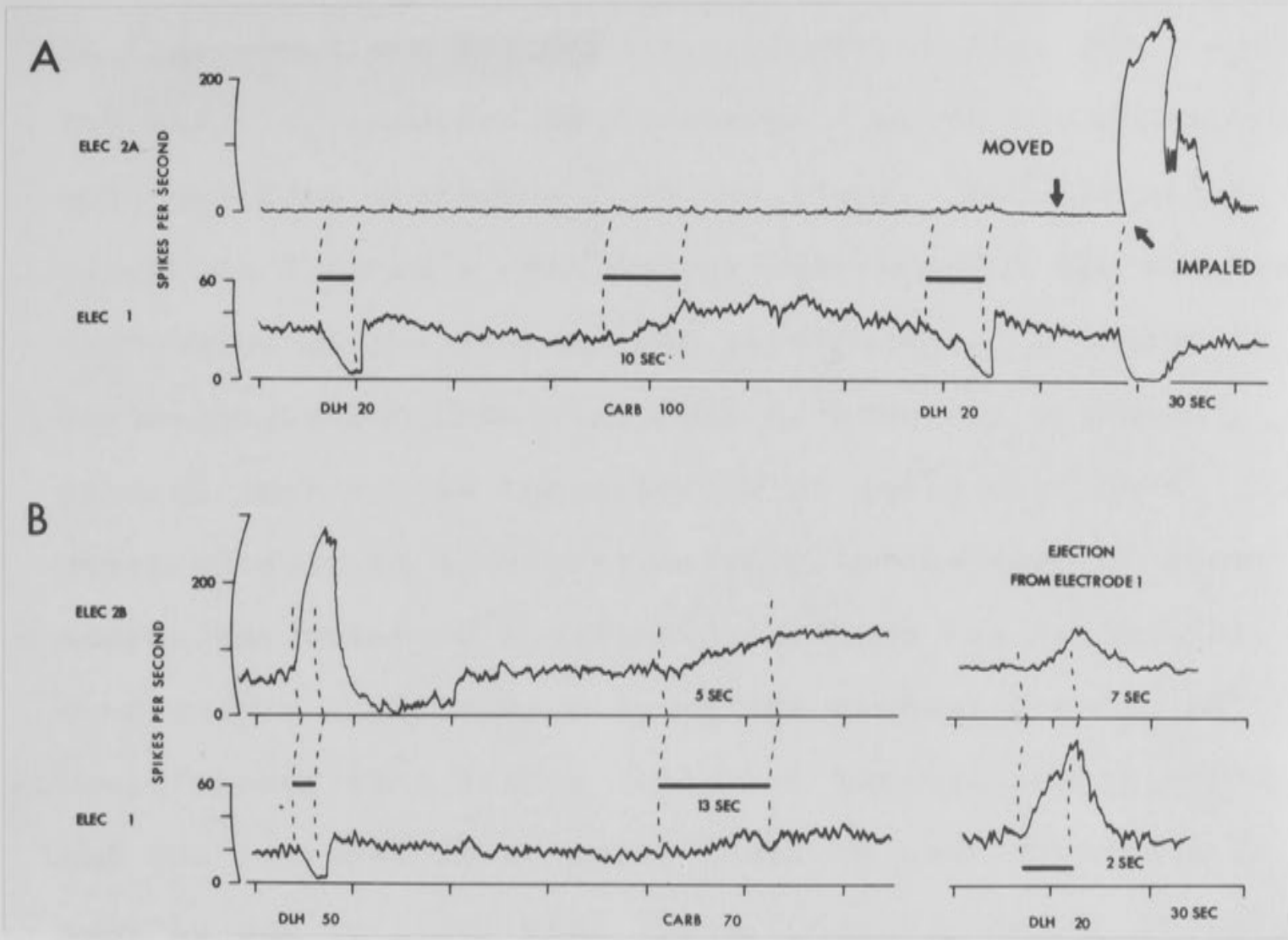

Fig. 26: Firing frequency records of the Purkinje cell near electrode "1" (lower traces) and of the activity recorded by electrode "2" transversely across the folium from "1" (see preceding Figure). Drug ejections were made from "2" at 200μ depth in A, and at 250μ in B. In each case, drug ejection is marked by the black horizontal bars between the frequency traces, the agent used and its ejecting current being indicated beneath the lower trace of each pair. The latent period of excitation is shown on each record.
At the end of record "A", electrode 2 was slowly moved with no drug ejection being made, but at the point shown a nearby basket cell was impaled, and discharged at a very high rate in consequence of this damage.
For full description of these experiments, see text.

in Fig. 26B, from the same experiment as Fig. 26A. Again the activity recorded by electrode 2 is on the upper trace and that from electrode 1 on the lower. DLH (50 nA) from electrode 2 fired a cell nearby and caused a short-latency depression of the P-cell near electrode 1. Ejection of carbaminocholine from electrode 2, however, produced a gradual increase in the activity of cells near both electrodes, with a shorter latency in the case of those nearer the point of ejection. As there was no initial decrease in the firing rate of the distant P-cell, it would appear that basket cells are insensitive to carbachol, and the increase in activity observed near electrode 2 must be due to other cell types, possibly nearby P-cells. When an attempt was made to demonstrate mutual interaction between the cells near each electrode by ejection of DLH from electrode 1, only a slower-onset excitation of the cells near '2' was observed. This was attributed to diffusion of the excitant, and the asymmetry of effect between electrodes 1 and 2 probably reflects an accident of microelectrode placement - relatively few basket cells near electrode 1 apparently end of the P-cells near electrode 2.

The rapidity of effects attributed to diffusion in these last experiments perhaps requires some explanation.

Because of the predominantly transverse spread of the dendritic trees of both basket and Purkinje cells, diffusion of a drug from any given point in the molecular layer will cause it to encounter the nearest portions of the dendrites of cells across the folium much sooner than it could reach the cell bodies of the same cells. Thus the inter-electrode distance, which is approximately the distance between the cell bodies of two cells, will be much greater than the distance actually traversed by the drug, and the latency of effects is correspondingly less than that expected on the basis of electrode separation.

(e) Actions on cells of the hippocampal cortex

(i) Carbaminocholine was more potent than ACh as an excitant both of hippocampal pyramidal cells and unidentified hippocampal neurones. Direct comparisons were made upon 12 cells in three preparations. The latency of onset of excitation by carbachol was comparable to that of ACh, but excitation was more persistent after carbaminocholine and lasted in some cases as much as a minute.

(ii) Acetyl-β-methylcholine was tried on only two neurones, and was found to be comparable in potency to acetylcholine but with a longer duration of action.

(iii) Nicotine was inactive upon three of five hippocampal cells sensitive to ACh, and was rather less potent than the remaining two cells.

It was not possible to elicit hippocampal waves due to the synchronized firing of many pyramidal cells (cf. Section III(d)) by the ejection of any of these cholinomimetics.

(f) Actions of neostigmine on cerebellar neurones

McCance and Phillis (1964a, b) have described a slowly-developing powerful excitation of cells in the granular layer of the cerebellum by electrophoretic ejection of anticholinesterases (neostigmine, BW 284 C51 and edrophonium). Both acetylcholine-protection anda direct excitatory action appear to play some part in this excitation. However, the same caution as before (Section V(g) above) must be applied in the acceptance of cell identification on the basis of histological lesioning technique. As suggested previously, these cells may in fact be Purkinje cells relatively remote from the recording electrode.

In the experiment illustrated as Fig. 27, an identified Purkinje cell was initially excited by ejection of ACh (40 nA) and DLH (3 nA). Minimal excitation was seen during the ejection of neostigmine with a current of 20 nA for 45 seconds, but there was only slight potentiation of the DLH-excitation one minute after the end of neostigmine administration. Ejection of neostigmine (40 nA) now caused

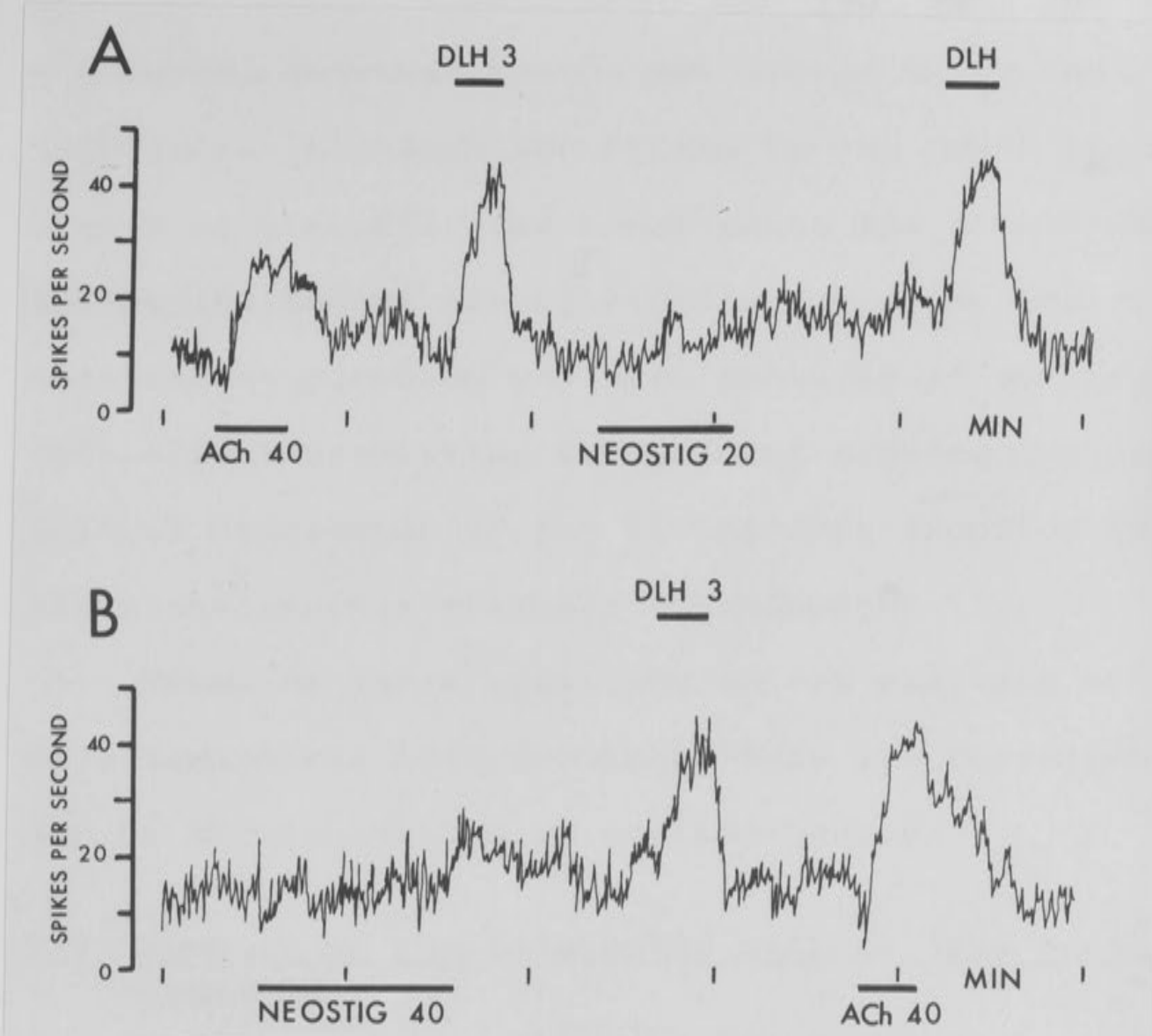

Fig. 27: Responses of a Purkinje cell to ejection of acetylcholine (ACh, current of 40 nA), DL-homocysteic acid (DLH, 3 nA) and neostigmine (20 and 40 nA). The frequency traces A and B are directly continuous. Further description in text.

Ordinate: Firing frequency (spikes/second).

Abscissa: Time in minutes.

a delayed, somewhat feeble and irregular excitation of this cell. Although the firing by the amino acid just over a minute after the neostigmine was almost unaffected, the excitation by ACh $2\frac{1}{4}$ minutes after the end of the neostigmine ejection was both potentiated and prolonged, this effect persisting for several minutes further. The initial depression of the firing rate produced by acetylcholine was slightly accentuated.

Thus, on these cells the direct excitant effect of neostigmine was less prominent than its potentiation of ACh by the inhibition of cholinesterase.

(g) Summary of cholinomimetic actions upon cortical neurones

Many features of the action of these substances have already been discussed above, but for convenience a summary is given in Table VII. dl-Muscarine and carbaminocholine were more potent than acetylcholine upon all the cholinoceptive cortical neurones tested, acetyl-β-methylcholine and nicotine were comparable in potency with ACh, and propionylcholine was considerably weaker. In general, all the sensitive cortical neurones behaved similarly towards these compounds, only minor variations in the order of potency being found between Betz, Purkinje and hippocampal pyramidal cells. It has not been possible

to demonstrate excitation of either basket cells or granule cells by any of the cholinomimetics tried.

When compared with the identified Betz and Purkinje cells, the 'insufficiently characterized' groups of cortical neurones show a significantly lower proportion of units sensitive to ACh and related compounds, but the behaviour of such members as are sensitive is closely similar to that of the corresponding 'identified' cells. However, only relatively major variations may be expected to reveal themselves in a short series of experiments such as the present.

The slow onset and delayed offset of excitation by cholinomimetics and the greater activity by muscarinic than nicotinic substances are in contrast to the behaviour observed with Renshaw cells (Curtis and Eccles, 1958; Curtis and Ryall, 1965a-c) and the 'intermediate' properties of thalamic neurones (Andersen and Curtis, 1964b). The functional significance of this behavioural pattern will be discussed later (Section XI(b)) in the light of the histochemical evidence produced by other workers and the results obtained with acetylcholine antagonists (Sections VII and VIII).

SECTION VII - ATROPINE

(a) Introduction

Because of the tendency of cholinomimetic compounds with high muscarinic activity to be more potent than nicotinic compounds on cortical neurones (Section VI), it was of interest to use atropine as an acetylcholine-antagonist to establish the muscarinic nature of the receptors (Dale, 1914). Furthermore, the effect of this antagonist upon synaptic firing of the various cortical neurones might well be expected to reveal participation of ACh in some specific synaptic pathway. In the cerebellum, for instance, there are two known monosynaptic excitatory pathways to the Purkinje cell, either by local surface stimulation which activates the parallel fibres (Dow, 1949; Andersen, Eccles and Voorhoeve, 1963), or by way of the climbing fibres originating in the inferior olive (Szentagothai and Rajkovits, 1959; Eccles, Llinas and Sasaki, 1964). In the latter case however, the synaptic activation of the P-cell is so intense that ACh-antagonists may conceivably fail to block the response completely even if transmission be cholinergic (cf. the resistance towards dihydro-β-erythroidine of the initial spikes of the Renshaw cell response to motor-axon collateral

activation - Curtis and Eccles, 1958; Curtis and Ryall, 1965c).

When ejected electrophoretically by relatively large currents, atropine has been shown to exert a non-specific 'local anaesthetic' action both upon Renshaw cells and other, non-cholinoceptive, spinal neurones (Curtis and Phillis, 1960). However it was anticipated that an additional specific reduction in the sensitivity of cortical neurones towards ACh should be demonstrable in view of the muscarinic nature of the receptor on these cells, which had been confirmed by the results obtained with intravenous atropine sulphate (see below).

(b) Systemic administration of atropine

(i) Effect upon cerebral cortical neurones

When injected intravenously in doses of 0.1 to 2.0 mg./kg., atropine reduced the responsiveness of cholinoceptive cortical neurones towards acetylcholine, but did not reduce that towards the amino acids, nor the 'background' spontaneous firing of the few units tested. These features are illustrated in Fig. 28, in which a neurone in an unanaesthetized cerveau isolé cat was initially excited at 55-60 spikes per second by 12 nA of DL-homocysteic acid, and at a slightly lower frequency by 20 nA of acetylcholine. The spontaneous firing pattern

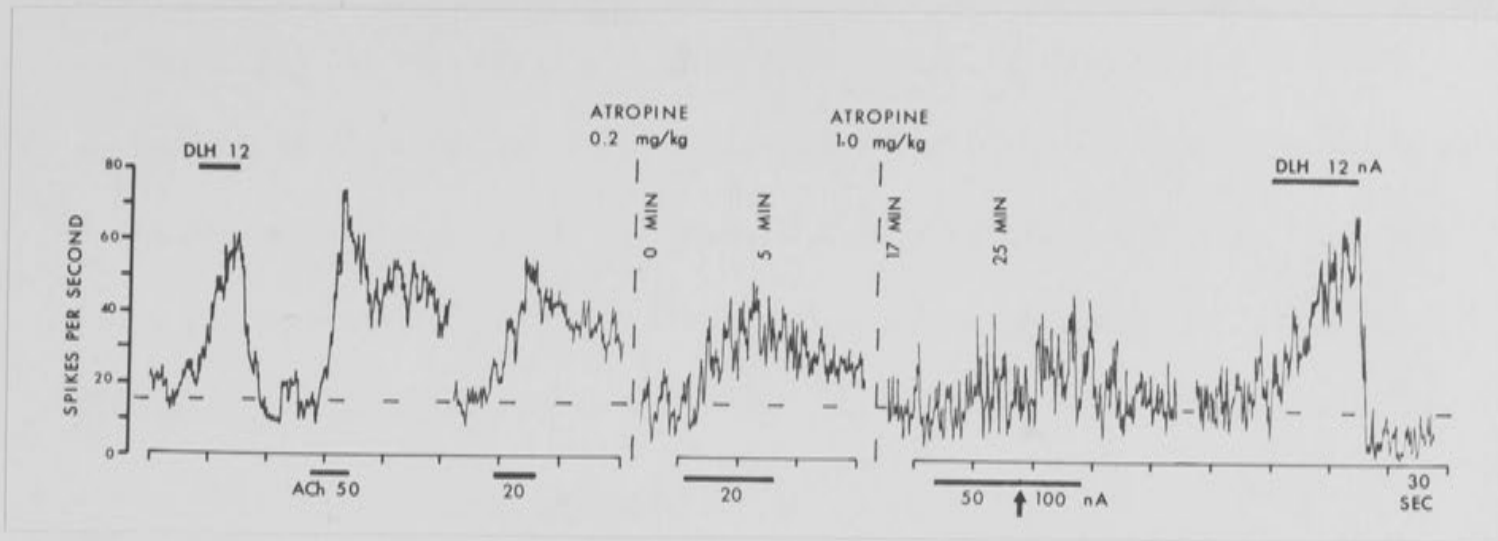

Fig. 28: Effects of intravenous atropine upon the chemical sensitivity of a Betz cell. Control responses to DL-homocysteic acid (12 nA) and to 50 and 20 nA currents ejecting acetylcholine are shown. At zero time, 0.2 mg/kg atropine was administered, and five minutes later ACh-ejection again tested. 17 Minutes after the first dose, another 1.0 mg/kg. atropine was given, and 7-8 minutes afterward currents of 50 nA ejecting ACh were almost ineffective on the cell's firing rate. At the arrow, the current ejecting ACh was doubled. The interrupted horizontal line at 15/second approximates the initial spontaneous firing rate. For further description, see text.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time scale (30 seconds).

was irregular, but averaged 10-20 spikes per second (in Fig. 28, the interrupted horizontal line at 15 per second is used to indicate an approximation to this initial rate). 0.2 mg./kg. of atropine sulphate was then given intravenously. The spontaneous background firing was apparently unaffected four minutes later, but a considerably longer period of ejection of ACh was now required for a maximal response, and this peak rate was reduced. A further dose of 1.0 mg./kg. of atropine followed, and 50 or even 100 nA of ACh then failed to fire the cell in a sustained manner at a rate above some 25 spikes/second. The background firing, though irregular, was still virtually identical with that during the control period, and DLH (which had also been tested at intervals between the injections of atropine not shown in the Figure) still fired the cell at some 65 spikes per second.

In other comparisons, however, Krnjević and Phillis (1963c) have found the background firing of cholinoceptive neurones to be reduced or abolished by atropine given intravenously.

(ii) Effect upon cerebellar Purkinje cells

Doses of as little as 0.1 mg./kg. of atropine sulphate intravenously substantially reduced the ACh-sensitivity of these cells without affecting their responsiveness to

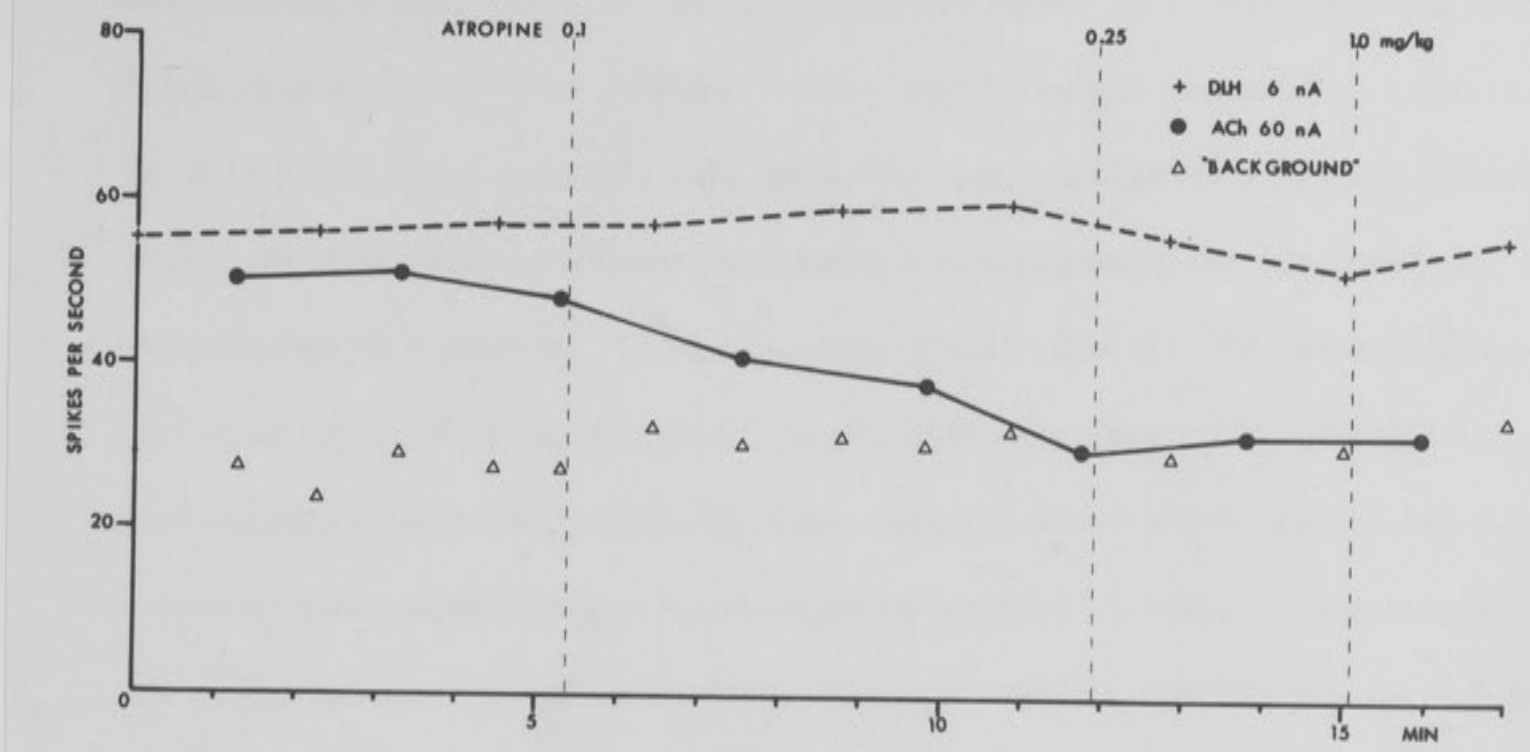

Fig. 29: Effects of intravenous atropine upon the chemical sensitivity and spontaneous firing of a Purkinje cell. Each point represents the maximum firing rate achieved by drug ejection (effects of DLH ejected by currents of 6 nA shown by +, while those of acetylcholine ejected with 60 nA are indicated by ●), or the "background" firing rate immediately prior to the start of each drug ejection (△). At the times indicated, successive doses of 0.1, 0.25 and 1.0 mg/kg of atropine sulphate were injected.

Ordinate: Peak firing responses (spikes/second).

Abscissa: Time in minutes.

DL-homocysteic acid, or any reduction in the spontaneous firing rate of the cell. One such experiment is illustrated in Fig. 29, in which 0.1 mg./kg. of atropine progressively reduced the ACh-response from 50 spikes per second to one undetectable above the 'background' level of some 30/second, but further doses of 0.25 and 1.0 mg./kg. of atropine did not significantly affect the amino acid firing rate nor reduce the spontaneous synaptic excitation. Recovery of the sensitivity of ACh was slow, taking half to one hour after atropine doses in this range.

(iii) Effect upon cerebellar evoked potentials

Six anaesthetized and two unanaesthetized cerveau isolé cats have been used for these experiments. Doses of 0.1 and 1.0 mg./kg. of atropine sulphate were given intravenously either singly or consecutively on thirteen occasions, and had no significant effect on the fields evoked by lateral reticular, external cuneate, juxta-fastigial or pontine nuclear stimulation. In various experiments, the evoked potentials were recorded either by a ball electrode resting lightly on the exposed cortical surface, or by a single-barrel NaCl-filled microelectrode at depths of 0.12 to 0.3 mm. beneath the surface, i.e. from points within the superficial dendritic region down to the level of the P-cell bodies. In a few trials,

attempts were made to record field potentials in the granular layer, down to 0.5 mm. depth.

Typical experiments are shown in Figs. 30 and 31. The complex field potentials evoked at 0.19 mm. depth by local stimulation of the surface of the vermis 2 mm. from the site of recording and in the longitudinal axis of the folium (LOCAL), by stimulation of the ipsilateral external cuneate nucleus (CUNEATE) and of the contralateral inferior olive (OLIVE) are shown in the topmost row of Fig. 30, while the control responses in another cat recorded at 0.27 mm. depth after juxta-fastigial (JF), external cuneate (CUNEATE) and lateral reticular (RETIC) stimulation are illustrated in the uppermost row of Fig. 31. The nuclei were approached stereotaxically with concentric needle electrodes as described in Section II(f).

The response to local stimulation included a negative wave with a latency of 2.7 to 3.0 msec., which was succeeded by a positive potential. On the peak of the negative wave, an occasional spike discharge was evident, but these were never seen during the positivity. Stimulation of the external cuneate nucleus gave a somewhat variable and complex negative potential with a mean latency of 4.6 msec., which was often followed by a smaller negative peak at 12 to 15 msec. latency (not clearly seen in Fig. 30, but more evident in the control responses of

Fig. 31). Olivary stimulation gave a negative potential peak, with a latency of 4 msec., and again followed by a small irregular later negativity. Juxta-fastigial stimuli evoke an initial peak with 3-4 msec. latency, and again a later second negative wave at 10 msec. (Fig. 31, JF), and stimulation of the lateral reticular nucleus gave a small initial peak at 3 msec., followed by a number of irregular negative waves superimposed upon a slowly decaying positivity (Fig. 31, RETIC).

The responses illustrated in Fig. 30B were filmed between 50 and 130 seconds after an intravenous dose of 1 mg./kg. of atropine sulphate, and some 9 minutes after an initial i.v. dose of 0.1 mg./kg. of atropine. Neither in this series, nor in those of Fig. 31B or 31C (after 0.1 and 1.0 mg./kg. atropine respectively) was there any significant change in the amplitude or latency of the initial negative potentials. Furthermore, in cases in which spike responses had been evoked by the stimuli during the control period, they were not abolished by the intravenous administration of atropine in these doses. Occasionally the second wave of the evoked responses appeared to be delayed or reduced in amplitude, but this was attributed to changes in the synchrony of arrival of the impulses which contribute to this late wave. In Fig. 31B, for instance, 0.1 mg./kg. of atropine appeared to

Fig. 30: Effects of intravenous atropine and dihydro-β-erythroidine on cerebellar responses evoked by electrical stimulation of the folial surface (LOCAL), the ipsilateral external cuneate nucleus (CUNEATE) and the contralateral inferior olive (OLIVE). After the control responses (A), a total of 1.1 mg/kg atropine sulphate was given intravenously and series (B) recorded, and finally 1.0 mg/kg of DHβE was given. The responses of series (C) were filmed 1-2 minutes after this latter drug. Further description in text. Negativity recorded upwards. Voltage calibration 0.5 mV.
Time scale: msec (all traces).

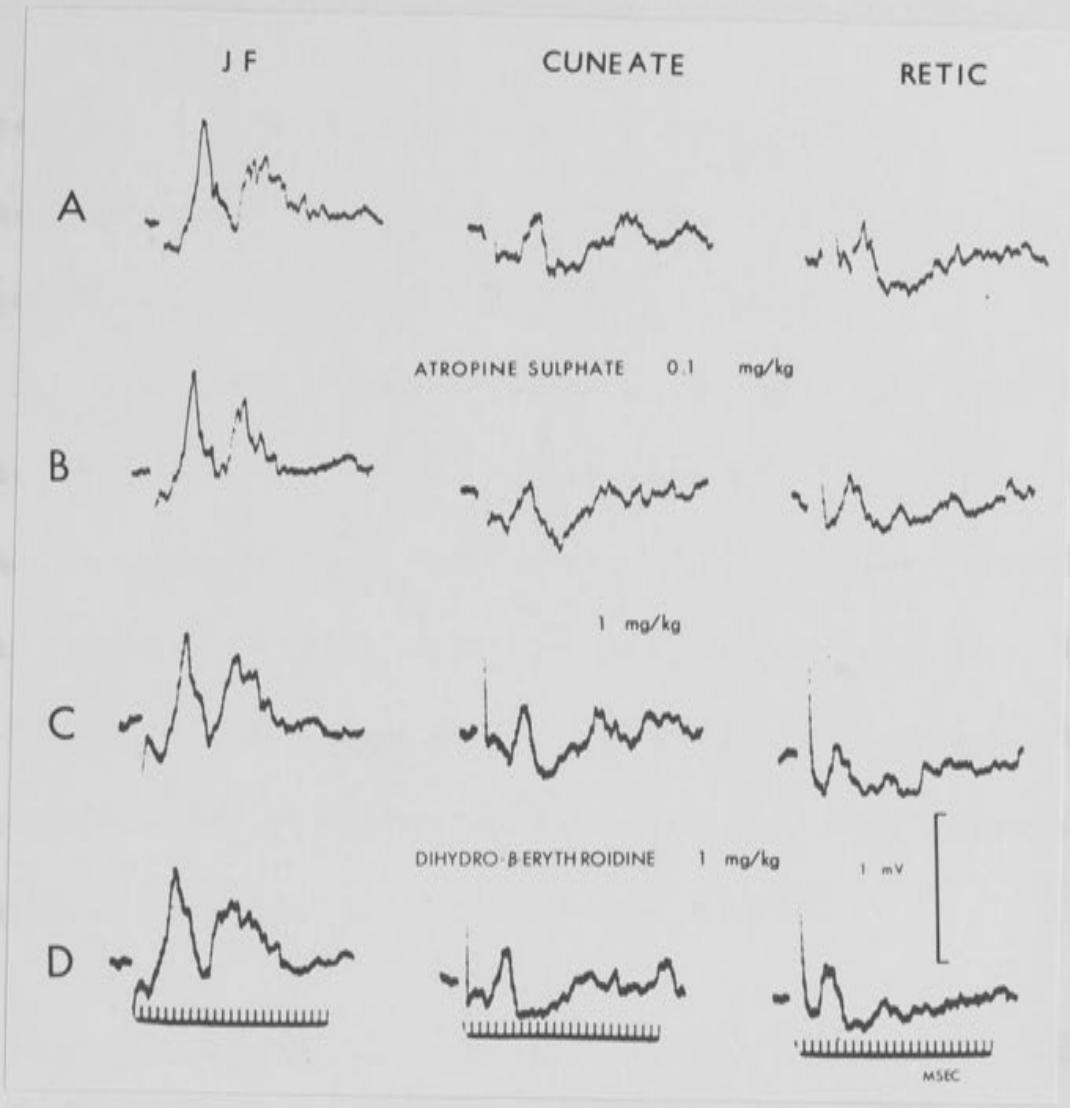

Fig. 31: Effect of intravenous atropine sulphate and dihydro-β-erythroidine on the responses evoked from juxtafastigial (JF), external cuneate (CUNEATE) and lateral reticular (RETIC) nuclei. Electrical stimulation was to the ipsilateral nucleus in each case. Following the control series (A), 0.1 mg/kg of atropine sulphate was given 2 min before series (B) was filmed. Subsequently, a dose of 1.0 mg/kg of atropine was given intravenously several minutes before series (C) was recorded, and finally 1.0 mg/kg of DHβE 2-5 minutes before the responses shown in (D) were taken. Voltage scale 1 mV; time scale in msec (all traces).

reduce the later component of the response to stimulation of the external cuneate nucleus, but 1 mg./kg. of atropine subsequently restored it! (Fig. 31C).

In two other experiments, 1 mg./kg. of atropine produced a slight (10-15 per cent) reduction in the size of the response to local parallel-fibre stimulation, but did not prevent the appearance of spikes due to Purkinje cell firing. It was never possible to demonstrate either substantial or prolonged effects of atropine upon any of the synaptic inputs which were tested.

(c) Electrophoretic administration of atropine

(i) Effects on Betz cells. In Fig. 32, the responses of a Betz cell to alternate ejection of acetylcholine (a current of 50 nA) and DL-homocysteic acid ($7\frac{1}{2}$ nA) are plotted, the magnitudes of these ejecting currents having been adjusted to produce control responses of similar frequency (55-70 spikes per second) to both substances. When atropine was allowed to diffuse from a 0.1 M solution of the sulphate in another barrel of the micropipette (Atropine 0 nA) the acetylcholine-sensitivity of the cell decreased slightly, but the firing produced by DLH was not consistently affected. Atropine was then passed electrophoretically by a current of 5 nA and the firing rate during ejection of ACh was reduced to 25/second, a value

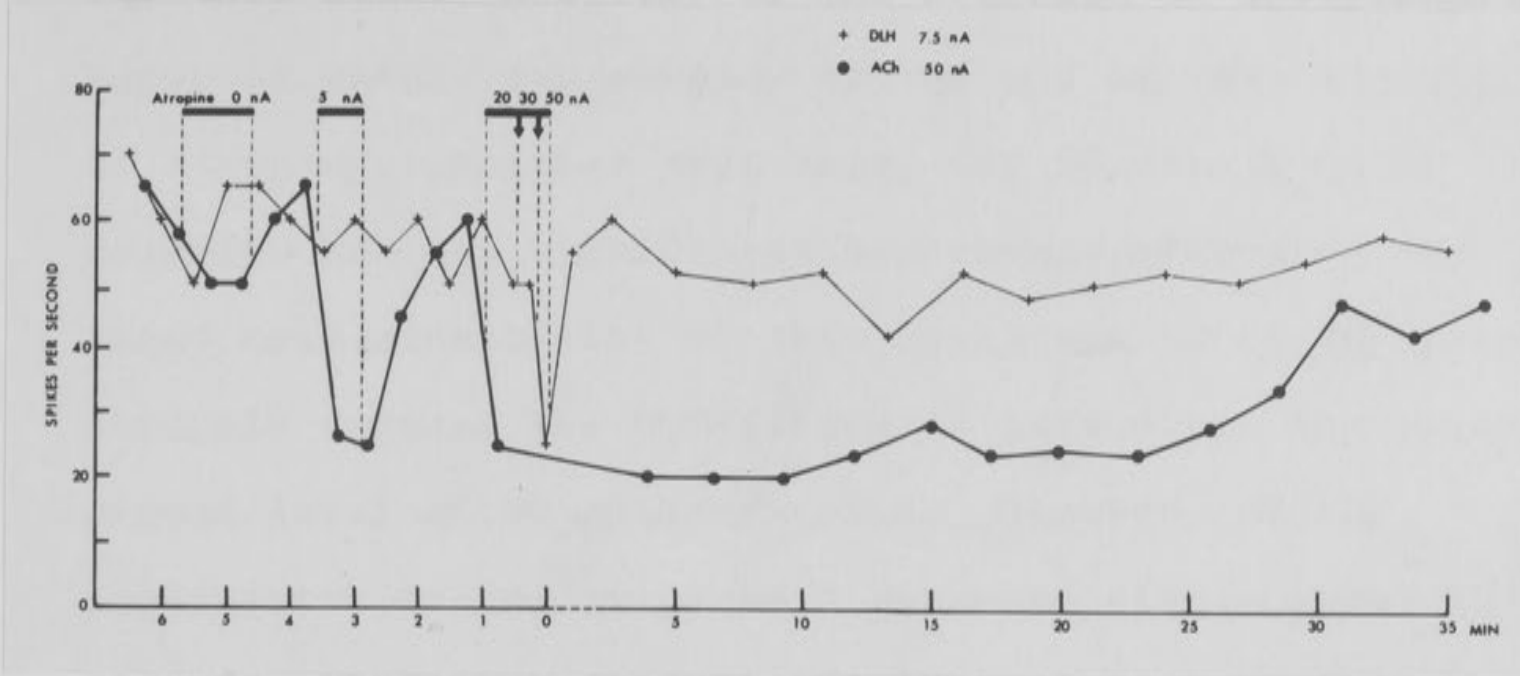

Fig. 32: Effects of electrophoretically ejected atropine upon the firing of a Betz cell by DL-homocysteic acid (DLH, 7.5 nA, +) and acetylcholine (ACh, 50 nA, ●). The peak firing rates achieved during alternate ejections of the two excitants are plotted, and during the periods shown by the black horizontal bars, the current retaining atropine was terminated (Atropine 0), and free diffusion from its 0.1 M solution permitted. Later, atropine was ejected as a cation by currents of 5 nA, and finally by a current which was raised successively from 20 to 30 and finally to 50 nA. Further description in text.

Ordinate: Peak firing frequency (spikes/second).

Abscissa: Time in minutes (note change in scale at origin).

very little greater than the spontaneous firing level for this cell. Recovery of the response to acetylcholine occurred within two munutes of the end of this ejection of atropine. Neither this dose, nor 20 nor 30 nA of atropine had any significant depressant effect on the amino acid sensitivity of this cell, but 50 nA of atropine abruptly reduced the DLH-firing to just above the background level of 20 spikes/second. Recovery of the sensitivity to the amino acid occurred within about 15 seconds, whereas a gradual and incomplete recovery of the firing by acetylcholine required over 35 minutes. In other experiments, the recovery time for ACh-firing varied from 25 to over 40 minutes.

Despite the profound and prolonged depression of the response to locally ejected ACh by as little as 5 nA of atropine, and the 'local anaesthetic' action exerted on the portion of cell membrane nearest the electrode tip by the ejection of 50 nA of atropine, as shown by the failure of DLH to excite the cell, it was not possible to demonstrate any substantial contribution to the 'background' excitation of these cells which could be blocked by electrophoretically ejected atropine.

(ii) Effects on cerebellar Purkinje cells. When atropine was ejected locally near P-cells, somewhat larger currents were necessary to reduce the ACh-firing than were

required in the case of Betz cells, and it was as a rule impossible to produce a definite antagonims to excitation by ACh without also diminishing DLH-evoked firing.

Two typical experiments are illustrated in Figs. 33 and 34. In Fig. 33, comparable firing rates of 50-55/ second were evoked by currents of 5 nA of DLH and 100 nA of acetylcholine. Atropine was then ejected, a current of 5 nA having no apparent effect on ACh firing of the cell. When the current was increased to 10 nA, atropine did reduce the sensitivity of the cell to acetylcholine. However, when the atropine ejection was discontinued, both chemically-evoked responses were increased, suggesting some non-specificity of action even with this dose of atropine. A current of 20 nA which ejected atropine definitely produced a non-specific (perhaps local anaesthetic-like) effect on both the acetylcholine and amino acid induced excitation.

In Fig. 34, although 10 nA of atropine (ejected from a 0.1 M solution) reduced the ACh-firing without altering the response to DLH, increasing the current which ejected atropine to 20 nA again markedly reduced excitation by both substances. In general, a dose of atropine only twice that which reduced ACh-firing would also diminish the effectiveness of amino acid excitation (compare the

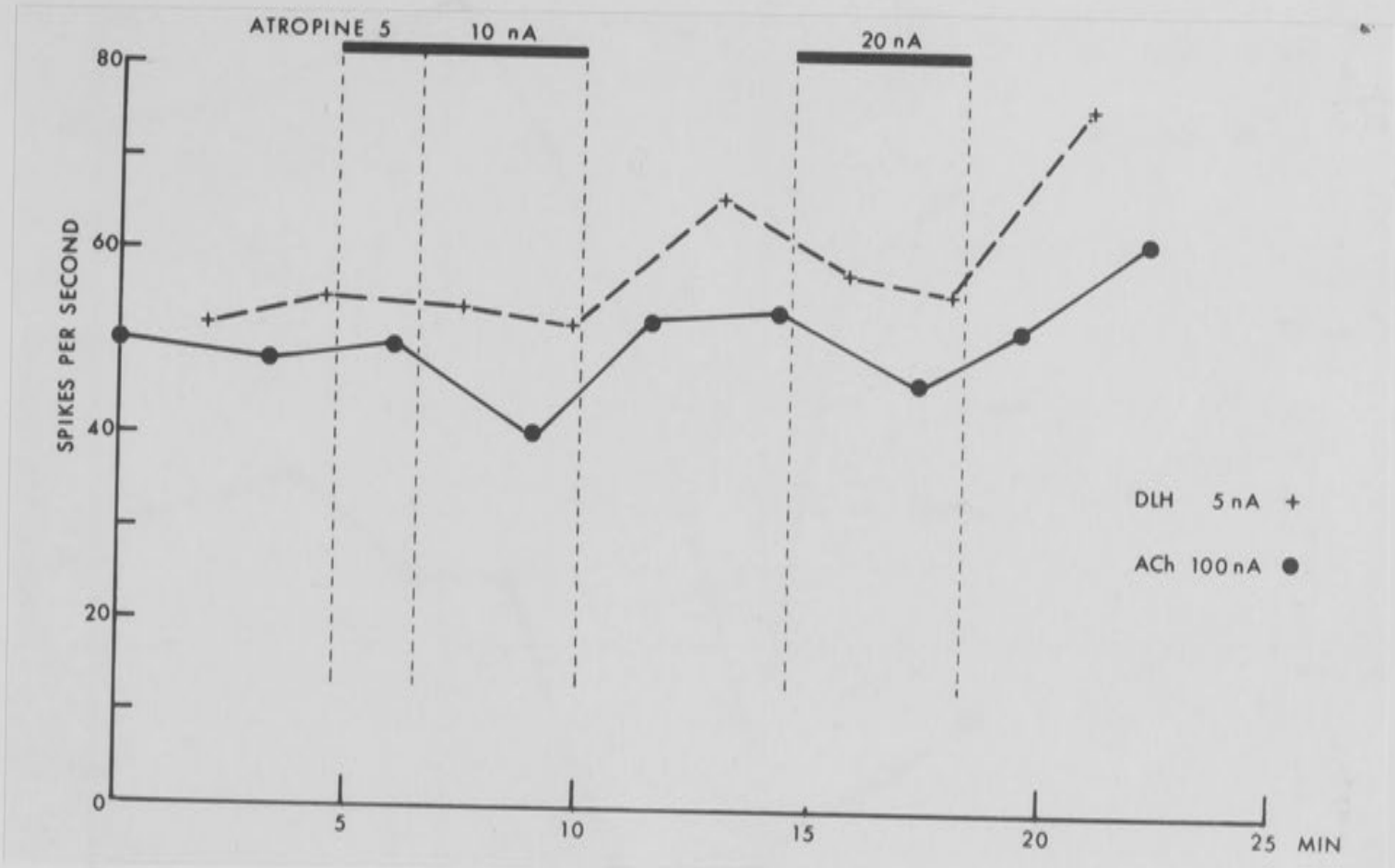

Fig. 33: Effects of electrophoretically ejected atropine (currents of 5, 10 and 20 nA) upon chemical excitation of a Purkinje cell. The peak rates evoked by alternate ejection of DLH (5 nA, +) and acetylcholine (ACh, 100 nA, ●) are plotted, and ejection of atropine by cationic currents was performed during the times shown by the black horizontal bars.

Ordinate: Peak firing rates (spikes/second).

Abscissa: Time in minutes.

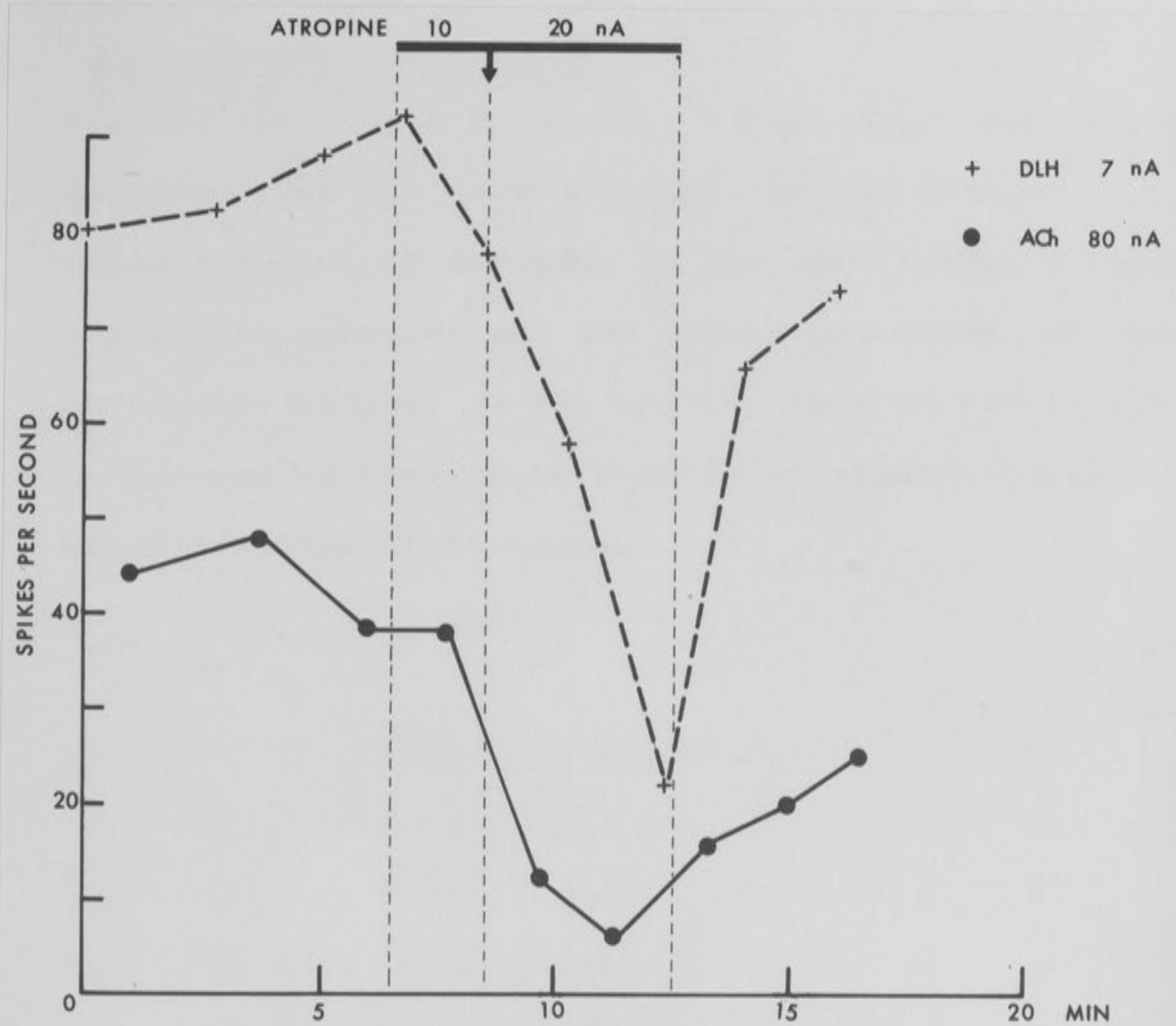

Fig. 34: Effect of electrophoretically ejected atropine on the chemical sensitivity of a second Purkinje cell. Peak firing frequencies elicited by alternate ejection of DLH (7 nA, +) and acetylcholine (ACh, 80 nA, ●) are plotted, and during the period shown by the horizontal bar, atropine was ejected by a cationic current of 10 nA, doubled at the arrow.

Further description in text.

Ordinate: Firing frequency (spikes/second).

Abscissa: Time in minutes.

tenfold ratio upon Betz cells - Fig. 32). However, as expected from the lack of effect of intravenous administration of atropine on the spontaneous firing of cerebellar neurones and the evoked potentials of the cerebellar cortex, it was not possible to reduce the background excitation of P-cells by electrophoretic administration of atropine.

SECTION VIII - DIHYDRO-β-ERYTHROIDINE, GALLAMINE and d-TUBOCURARINE

Although the acetylcholine-responses of cortical neurones are predominantly muscarinic (Sections V-VII), there is evidence also of excitation by nicotinic compounds, and it was of interest to try compounds antagonistic to such actions.

(a) Systemic administration of DHβE

(i) Effects upon cerebral cortical neurones

Systemic DHβE has been tested neither upon the chemical sensitivity of Betz cells nor upon unidentified cortical neurones. However, in the light of the muscarinic character of the receptor upon these neurones, and the failure of locally-ejected DHβE to prevent the occurrence of excitation by ACh (see below), the systemic administration of this antagonist might well be expected to be without effect.

In one experiment on the recurrent inhibition of Betz cells, 0.2 mg./kg. of DHβE intravenously had no effect on the relationship between the firing frequency of the cell produced by an amino acid and the duration of the pause following a pyramidal tetanus (see also Section IX(a)i for further description of these experiments).

(ii) Effects upon Purkinje cells

Doses of 1-1.2 mg./kg of DHβE given either at once or in three or four smaller doses over a period of 10-15 minutes failed to affect the sensitivity of the cells tested to either acetylcholine or DL-homocysteic acid. One such trial is illustrated in Fig. 35. The spontaneous firing of the cells, and the effects produced synaptically by local surface stimulation of the parallel fibre system, both on and off-line, were also unaffected.

(iii) Effects upon cerebellar evoked potentials

Typical results are shown in the lower traces of Figs. 30 and 31. Following the administration of atropine, which had been without effect on any of the potentials evoked from the various nuclei (Section VII(a)iii above), doses of 1.0 mg./kg. of DHβE were given in each case. As with atropine, there was no profound or long-lasting effect upon any of the potentials. Even the slight reduction in the external cuneate response of Fig. 30C (filmed one minute after the DHβE had been given) had disappeared when the next series of responses were filmed two minutes later (not shown in Fig. 30). No effect at all of DHβE upon the response to external cuneate stimulation was seen in other experiments such as that of Fig. 31D.

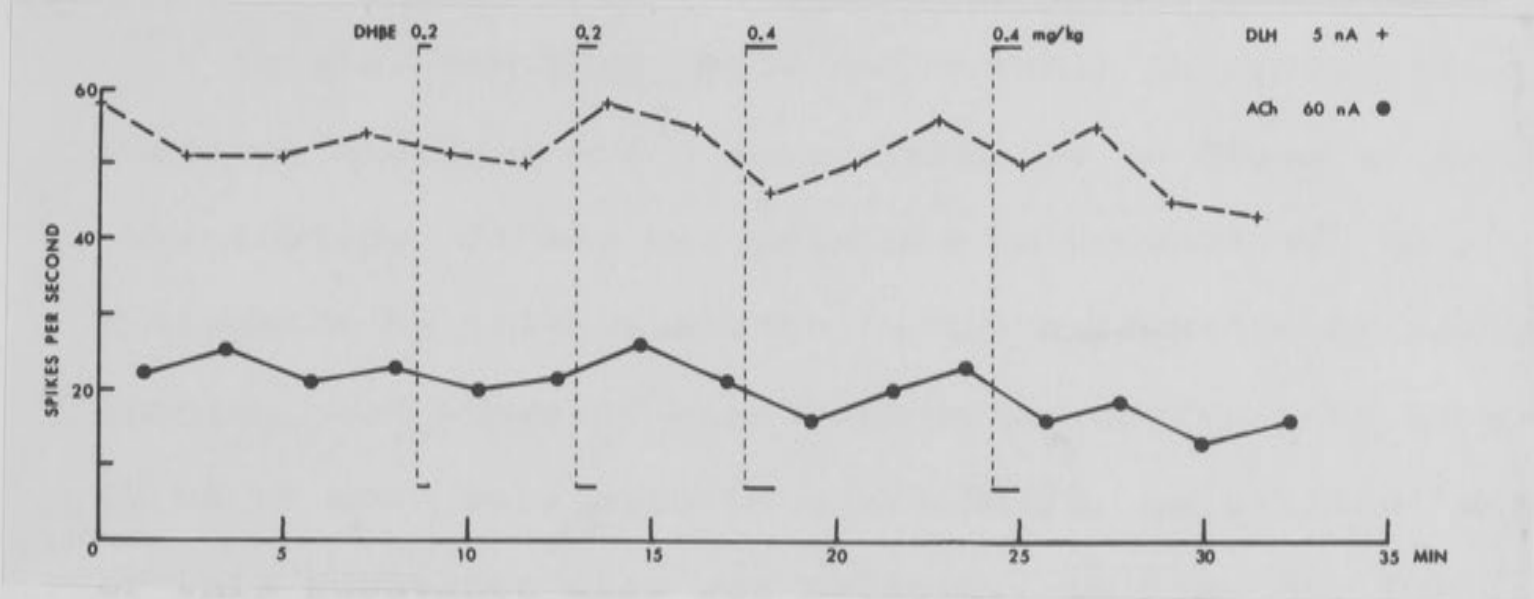

Fig. 35: Effect of successive intravenous doses of 0.2, 0.2, 0.4 and 0.4 mg/kg of dihydro-β-erythroidine given over a period of 17 minutes upon the chemical excitation of a Purkinje cell. The peak firing rates elicited during alternate ejections of DL-homocysteic acid (DLH, 5 nA, +) and acetylcholine (ACh, 60 nA, ●) are plotted as a function of time (scale in minutes).

(b) Electrophoretic administration of DHβE

(i) Effects upon pericruciate cortical neurones

In all, ten Betz cells and several unidentified cortical neurones which were sensitive to ACh-ejection were tested. It was not possible to demonstrate an antagonism by this substance to the excitation by acetylcholine, and wherever electrophoretic currents of some 20 nA or more were used to eject DHβE, an excitant action of this substance also was noticed. In Fig. 36, for instance, neither 10 nor 15 nA of DHβE had any apparent effect on the spontaneous or ACh-induced firing of this cell, which was a Betz cell with 1½ msec. latency to pyramidal stimulation. Substantially greater excitation was seen during the periods of ACh-ejection after the DHβE-current had been increased to 30 nA. Following termination of the DHβE, partial recovery occurred within 1-2 minutes, but unfortunately the cell was thereafter lost. In other experiments in which DHβE actually fired the cell, the time-course of excitation by this substance appeared to be fairly slow in both onset and offset, persisting for ½-1 minute after the end of the ejection.

(ii) Effects upon Purkinje cells

In three cases out of the ten experiments performed it was possible to show an antagonism by DHβE to the

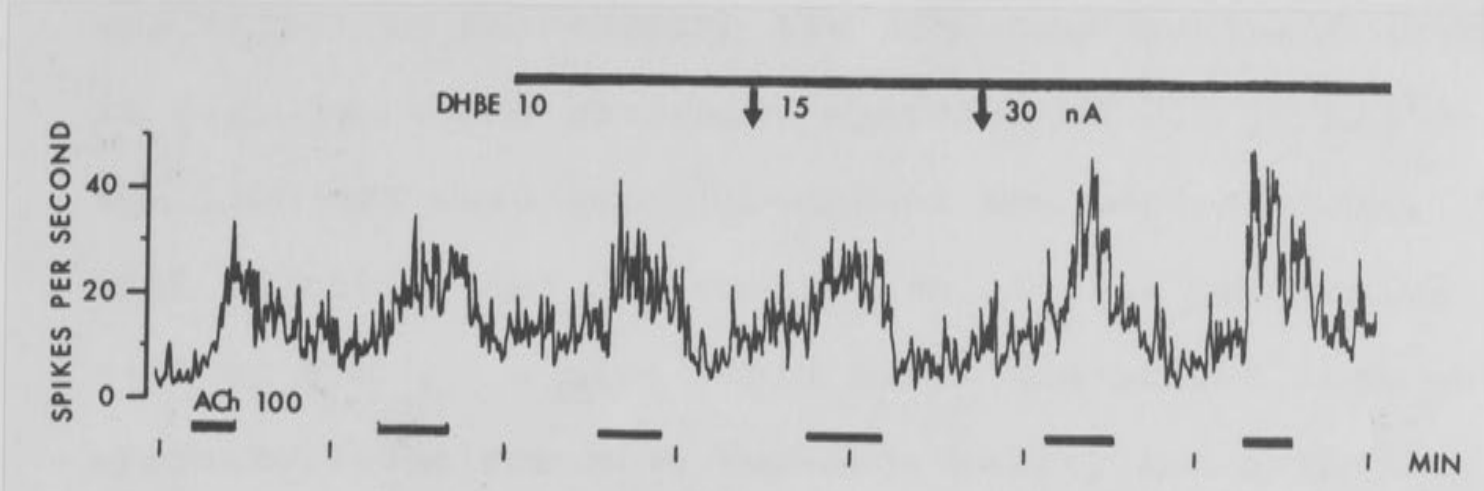

Fig. 36: Electrophoretic ejection of dihydro-β-erythroidine (DHβE) and the excitation of a Betz cell by acetylcholine (ACh, current of 100 nA during each of the lower bars). The long horizontal bar above the frequency-trace signals ejection of DHβE by a current of 10 nA, increased successively to 15 and 30 nA at the arrows. Further description in text.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time in minutes.

action of ACh upon Purkinje cells, but in the majority of trials there was no apparent reduction in the cells' sensitivity to ACh. One of the trials in which DHβE was effective in reducing the ACh-response is illustrated in Fig. 37, where alternate ejection of DLH (8 nA) and ACh (100 nA) were made throughout the period shown. It will be noted that the excitation of this particular cell by ACh had a more rapid onset and offset than was characteristic for most Purkinje cells, and a similar observation was made with the other two cells upon which DHβE successfully antagonized ACh-excitation.

After the control responses of Fig. 37 had been recorded, 24 nA of DHβE was ejected from another barrel of the micropipette, and a progressive decline in the response to ACh-ejection ensued over four minutes. About two minutes after the commencement of DHβE administration, however, a direct excitant action of this compound was noted, and this discharge persisted for several minutes after the end of the DHβE ejection. Following termination of the antagonist, the ACh-responses returned to control levels over some 4-5 minutes.

In the other seven cells tested, DHβE did not block the acetylcholine-produced firing, but a direct excitation by DHβE (currents of 10-50 nA), or a facilitation of the firing produced by either ACh or DLH, was common.

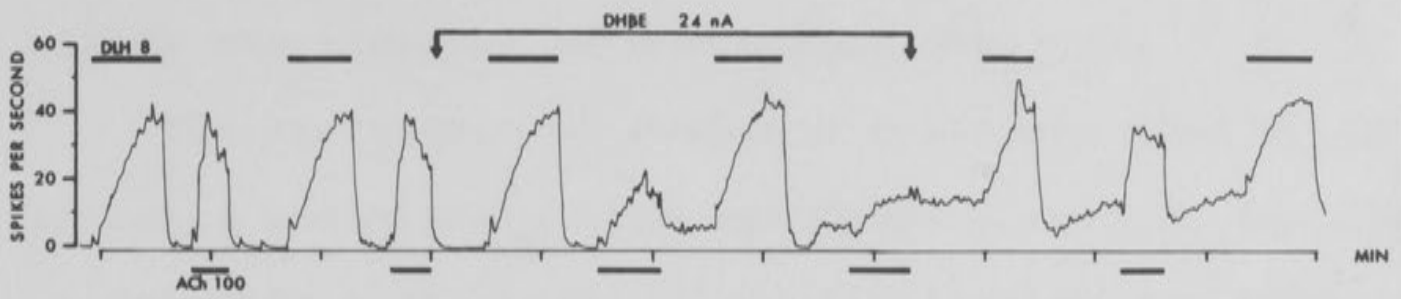

Fig. 37: Interaction of electrophoretically ejected DHβE with the chemical excitation of a Purkinje cell. Alternate ejections were made of DLH (8 nA, bars above frequency trace) and acetylcholine (ACh 100 nA, during the periods marked by the bars below the frequency trace). Ejection of DHβE with a cationic current of 24 nA took place in the period marked by the uppermost signal. Further description in text.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time in minutes.

(c) Actions of d-tubocurarine

This substance caused excitation of all five Purkinje cells, and two of four unidentified cerebellar neurones, upon which it was ejected. d-tubocurarine was not tested as an antagonist of acetylcholine-induced excitation in either the cerebral or cerebellar cortices.

The excitation of Purkinje cells was most apparent in cells which had a high spontaneous rate of discharge, or in which a 'background' depolarization was produced by the continuous ejection of DLH or carbaminocholine.

Results from one cell, in which a continuous ejection of DLH ($2\frac{1}{2}$ nA) evoked a low background discharge rate of 5-10 spikes per second, are shown in Fig. 38. Both acetylcholine (80 nA) and d-tubocurarine (40 nA) produced excitation when the cationic ejecting currents had ceased. In this case the excitation due to d-tubocurarine was greater and more prolonged than that due to ACh, but in other cells in which the depressant effects of the cationic currents were less prominent, d-tubocurarine produced a slowly increasing rate of firing during the entire period of ejection (up to 50 seconds), and had an offset time of up to two minutes.

(d) Systemic administration of gallamine

In a total of 63 cats, gallamine triethiodide (Flaxedil, May and Baker, Ltd.) was given intravenously in

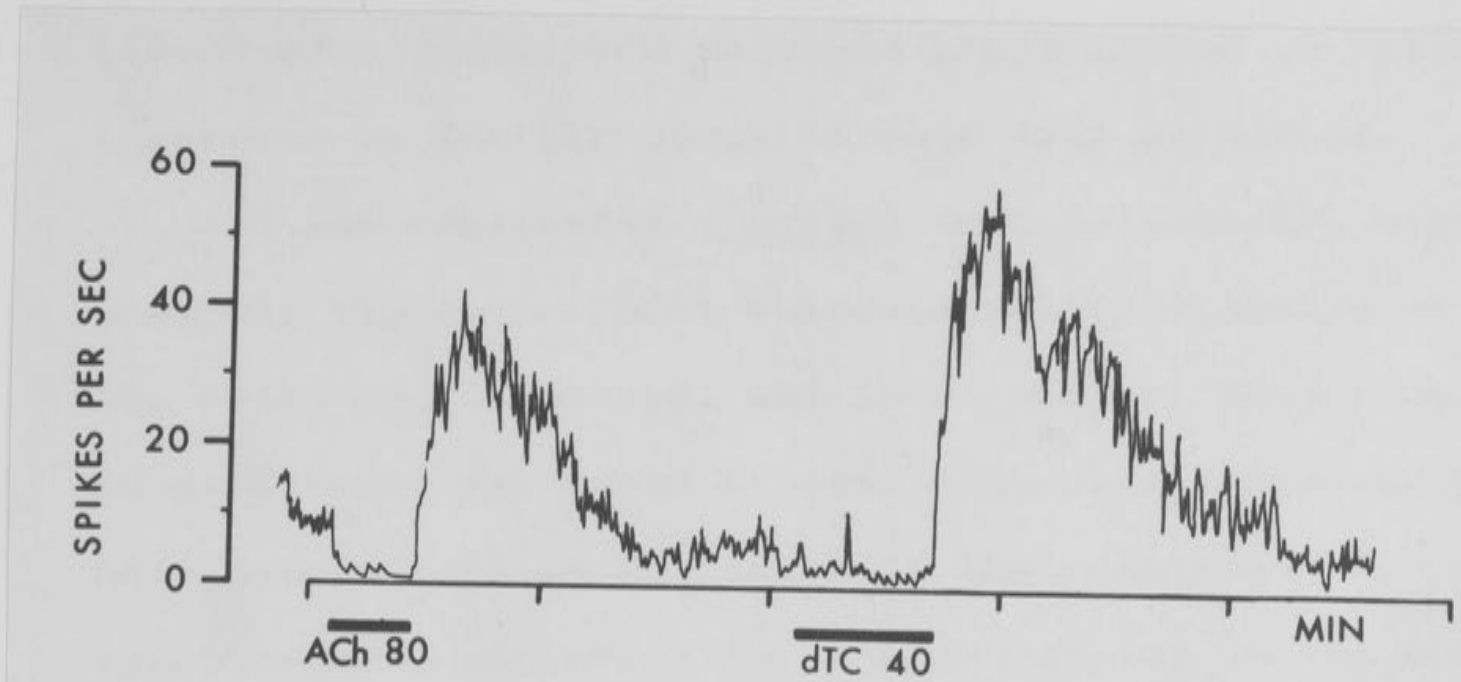

Fig. 38: Excitation of a Purkinje cell by electrophoretically ejected ACh (80 nA) and d-tubocurarine (dTC, 40 nA). Throughout the period shown, a small background depolarization of the cell was maintained by the ejection of DL-homocysteic acid by a current of $2\frac{1}{2}$ nA.
Ordinate: Firing frequency in spikes/second.
Abscissa: Time in minutes.

order to induce and maintain paralysis of theanimal during the experiments. The initial dose was 5-7 mg./kg. (10-20 mg. total), and this was supplemented at half-hour intervals by further doses of half this magnitude.

It was considered a priori that this bulky, highly charged, lipid-insoluble molecule would be unable to cross the blood-brain barrier, and in support of this expectation no difference was found between control animals and the cats paralysed with gallamine in the proportion of spontaneously active cells in each cat, or in the ratio of cholinoceptive units to the total number of active cells encountered. In addition, studies were made of the firing of single neurones in both the cerebral and cerebellar cortices by electrophoretically ejected ACh while doses of up to 4 mg./kg. of gallamine were injected slowly intravenously. Under these conditions, there was no apparent effect on the chemical responsiveness of the cell, nor on the spontaneous firing rate or the firing pattern. It would not appear, therefore, that the synchronizing effect of intravenous gallamine upon the electrocorticogram (Hodes, 1962) can be related to direct effects upon Betz cells (cf. Krnjević and Phillis, 1963c, page 336). However, it is conceivable that gallamine has an effect upon ACh-controlled cells in other areas where the blood-brain barrier is more permeable.

(e) Electrophoretic ejection of gallamine

Twenty cerebral cortical neurones and three Purkinje cells were tested using currents of 10-30 nA to eject the drug. A characteristic pattern of excitation was observed in both cholinoceptive and non-cholinoceptive units of both the cerebrum and the cerebellum. This consisted of bursts of high-frequency firing (up to 500 or 600/second for about a tenth of a second) which recurred at intervals of approximately one second. This excitation was sometimes superimposed on the background firing of the unit, but was accentuated by the concurrent ejection of either ACh (Fig. 39, a Betz cell) or DLH (Fig. 40, a non-cholinoceptive cerebellar neurone). There was no evidence of a specific antagonism of gallamine towards ACh-excitation, but on a few occasions the combined ejection of two excitants led to a very rapid depolarization block of the cell (cf. Krnjević and Phillis, 1963c).

This excitant action of gallamine, exerted on the cells irrespective of their sensitivity to ACh, is essentially similar to that described for neurones in the medulla (Salmoiraghi and Steiner, 1963), thalamus (Andersen and Curtis, 1964b), and spinal cord (Curtis and Ryall, 1965b).

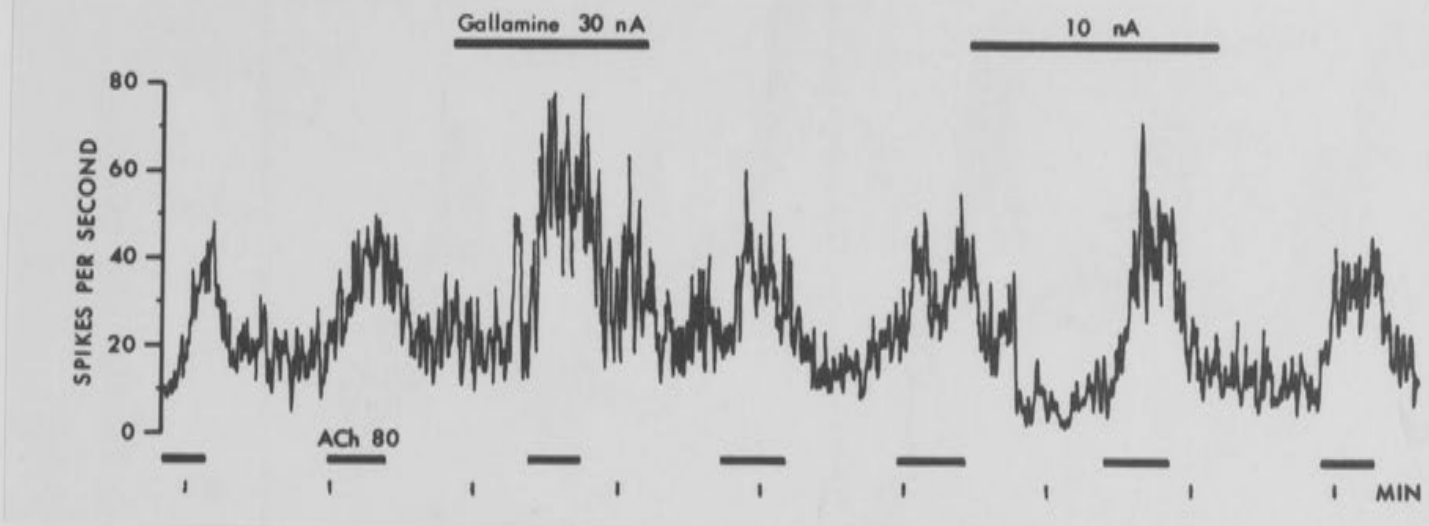

Fig. 39: Effect of electrophoretic administration of gallamine (currents of 30 and 10 nA during the upper signals) upon the spontaneous and ACh-evoked firing of a Betz cell. Currents of 80 nA were used to eject acetylcholine during each of the lower markers.
Ordinate: Firing frequency (spikes/second).
Abscissa: Time in minutes

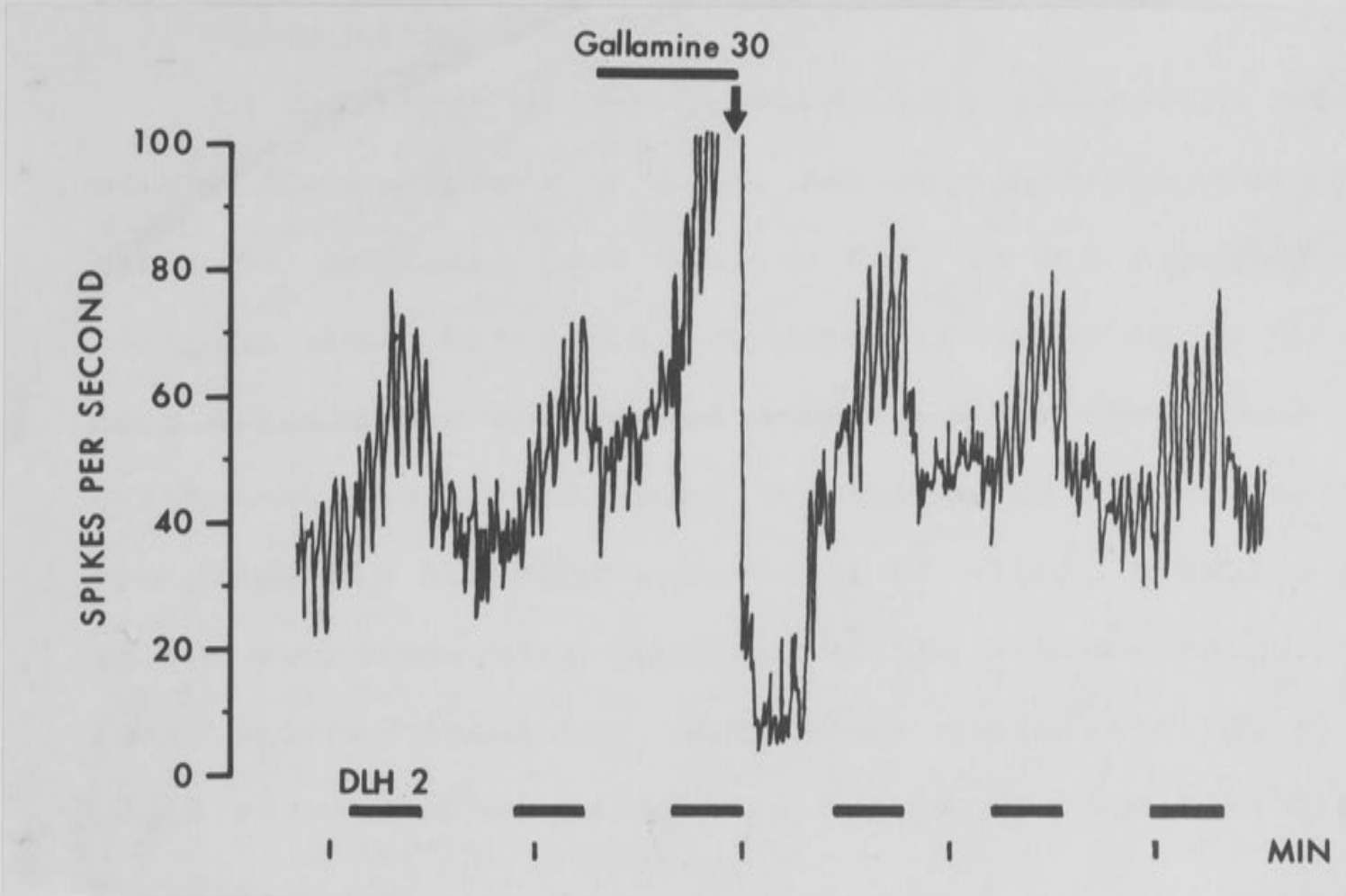

Fig. 40: Excitation by electrophoretic administration of gallamine (current of 30 nA) of a non-cholinoceptive unit in the cerebellar cortex. Repeated small doses of DL-homocysteic acid (DLH, 2 nA) were ejected during the lower horizontal markers. At the point shown by the arrow, the spike changes of "depolarization block" were noted.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time in minutes.

(f) Discussion of the results obtained with ACh antagonists

In the light of the predominantly muscarinic nature of the ACh-receptors on Betz, Purkinje and hippocampal pyramidal neurones (see Section VI), it was expected that atropine would block the responses of these cells to ACh more effectively than would dihydro-β-erythroidine, d-tubocurarine or gallamine triethiodine, all of which are primarily blocking agents for nicotinic synapses such as the neuro-muscular junction of the Renshaw cell. In fact, neither local nor intravenous administration of DHβE reduced ACh-sensitivity, except for a minority of P-cells.

The excitant action of the 'curariform' agents when ejected electrophoretically may reflect differences in the configuration of the receptor site on cortical neurones from that of nicotinic junctions. Drugs which are able to compete with and block the action of ACh at one type of receptor may activate another type, because variation in the disposition of active sites within the receptor area enables the drug to 'fit' differently upon them. However, the excitation by gallamine may not involve such interaction with ACh-receptors, as it has been reported upon all types of neurone so far examined, irrespective of their sensitivity to ACh (Salmoiraghi and Steiner,

1963; Andersen and Curtis, 1964b; Curtis and Ryall, 1965). In the present series, the characteristic excitation by gallamine completely overshadowed its weak atropine-like action (Riker and Wescoe, 1951; Laity and Garg, 1962), although Krnjević and Phillis (1963c) report a 'specific and fairly consistent' blocking action on the ACh-firing of cortical neurones.

When blocking agents capable of crossing the 'blood-brain barrier' (such as atropine or DHβE) are given intravenously, their distribution around any particular neurone may be taken as approximately uniform over the whole cell surface. In contrast, micro-electrophoresis yields very high drug concentrations at points near the electrode tip, thereafter falling off inversely with the distance from the ejecting point (on the assumption that the medium surrounding the electrode is homogeneous) (Curtis, Perrin and Watkins, 1960). If, as was assumed, the microelectrode tip had to be close to the cell soma and large dendritic trunks in order to record extra-cellular spikes of reasonable size, these regions of the cell will also receive the highest concentration of any drug being ejected.

Both locally-ejected and intravenous atropine were effective in producing a prolonged reduction in the sensitivity of Betz cells to ACh-ejection, though not in

reducing the spontaneous firing of the cells. This would suggest either that the cholinergic contribution to the synaptic bombardment of Betz cells is small, or that the cholinergic synapses are located so far out on the dendrites that electrotonically propagated depolarization from them normally contributes little to the generation of spikes at the initial segment. The fact that locally-ejected atropine can block the responses of these distant receptors to electrophoretic administration of ACh is an indication of their affinity for the antagonist, which reaches them only in low concentration. In this connection, it is of interest that Creuzfeldt and Lux (1964) have recently determined that excitatory synapses of Betz cells fall into two main groups, 'specific' e.p.s.p's being evoked by stimulation of the ventrolateral thalamic nuclei and apparently arising on the Betz cell body, whereas 'unspecific' e.p.s.p's (elicited by stimulation of the centre median) occur upon dendrites remote from the soma. These latter 'unspecific' endings appear to be concerned with alterations in the general level of Betz cell excitability.

In contrast to the prolonged depression of ACh-firing by both local and intravenous atropine on Betz cells, Purkinje cells show somewhat less susceptibility to this blocking agent. When atropine was ejected locally near

P-cells, it was difficult to separate ACh-antagonism from the non-specific local anaesthetic action (Curtis and Phillis, 1960), although intravenous administration of atropine did specifically reduce the cell's sensitivity to acetylcholine. These findings were taken to indicate a site relatively remote from the microelectrode for the ACh-receptors on these cells also - in order to eject enough atropine to diffuse out to and compete with ACh at the distant receptors, very high local concentrations were necessary, and these affected the cell membrane underlying the electrode tip, rendering it unsusceptible to DLH. The relative ineffectiveness of atropine on ACh-excitation of P-cells may be correlated with the variation of these cells from typical 'muscarinic' behaviour. Nicotine was rather more effective upon P-cells than on cerebral cortical neurones (e.g. Table VII), and a few P-cells showed short-latency excitatory responses to ACh, which were blocked by DHβE (Fig. 37).

Neither local nor intravenous administration of either atropine or DHβE had any significant effect upon the spontaneous firing of the cortical neurones tested, nor were these agents effective in altering the evoked potentials of the cerebellar cortex. The doses employed intravenously were adequate to block the sensitivity to ejected ACh (as tested on other cortical neurones in the

case of atropine, and on Renshaw cells in that of dihydro-β-erythroidine), and these negative findings cast some doubt on the proposed cholinergic innervation of the cerebral and cerebellar cortices. The presence of barriers around the synapses involved is unlikely, as these barriers would have to admit locally-ejected ACh though not the blocking agents. However, a sufficiently intense synaptic barrage (such as that of the climbing fibres upon Purkinje cells, or that responsible for the initial spikes of the Renshaw cell response) may be resistant to the blocking agents.

SECTION IX - OTHER PHARMACOLOGICALLY ACTIVE SUBSTANCES

A number of drugs which have been shown to mimic or block the actions of synaptic transmitters at other central sites have also been tested upon the responses of cortical neurones.

(a) Strychnine

(i) Systemic administration of strychnine

This substance is a powerful antagonist of post-synaptic inhibition in the spinal cord (Bradley, Easton and Eccles, 1953; Curtis, 1959, 1962b; see also reviews by Curtis, 1963, and by Eccles, 1964), but is ineffective in blocking the prolonged hyperpolarizing potentials of Purkinje cells which result from 'off-line' stimulation of the cerebellar cortex (Andersen, Eccles and Voorhoeve, 1963; Andersen, Eccles, Løyning and Voorhoeve, 1963).

Using the extraceullular ejection of DL-homocysteic acid as a means of exciting the cells, postsynaptic inhibitory actions upon Betz and Purkinje cells were studied. Local stimulation of the cerebral cortex (e.g. Krnjević, Randić and Straughan, 1964), or a short tetanus of six impulses at 100/second to the medullary pyramids will inhibit Betz cells for prolonged periods (Phillips,

1959), while a single local stimulus to the cerebellar surface 1-2 mm. away from the recording site in the same folium hyperpolarizes and inhibits Purkinje cells (Andersen et al., 1963). In general, it was possible to fire Betz and Purkinje cells over a range of frequencies by varying the dose of DLH ejected near them. Filmed records of the extracellular spike discharge were made, and the duration of the 'pause' in spontaneous or chemically-induced firing which followed an inhibitory stimulus was measured from these records. The pre-stimulus firing rate was also calculated from measurements of the interspike interval.

The duration of the pause was found to vary in an approximately inverse relationship with the rate of firing immediately preceding the stimulus (Fig. 41B). In a few cells, however, there was considerable variation in the length of the pause despite a relatively constant firing frequency (Fig. 41A). Intravenous strychnine was then given in doses of 0.1 to 0.6 mg./kg., and further records were made of the relationship between the firing-rate and the duration of the pause. There was no evidence of any effect of strychnine upon the curves obtained with either Betz or Purkinje cells, the overall scatter of points before and after strychnine being closely similar

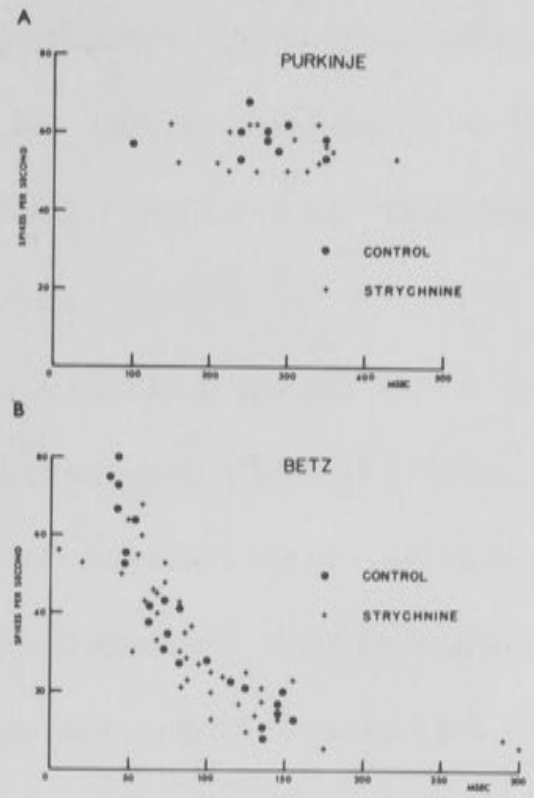

Fig. 41: A: duration of surface-evoked inhibition of a Purkinje cell plotted in relation to the spontaneous firing frequency before (●) and after (+) the intravenous administration of strychnine hydrochloride (0.33 mg/kg).

B: duration of the "recurrent" inhibitory pause in firing of a Betz cell following six stimuli at 100/second to the medullary pyramids. Various amounts of DLH were ejected electrophoretically to alter the firing rate of this cell, and again points shown "●" are controls, and those shown "+" were obtained after intravenous strychnine hydrochloride (0.6 mg/kg).

(Fig. 41). In fact, the longest duration of the 'pause' in Betz cell firing (300 msec.) was noted after 0.6 mg./kg. of strychnine. It was therefore concluded that strychnine had no effect upon the (postsynaptic) recurrent inhibition of Betz cells nor the basket cell inhibition of Purkinje cells.

A contrary finding with Betz cells has been reported by Suzuki and Tukahara (1963), but their method of testing by measurement of antidromic invasion may not completely have excluded excitatory influences of strychnine which increased the background synaptic drive on the neurone. In other experiments, Stefanis and Jasper (1965) found that 0.5-1 per cent strychnine applied topically to the pericruciate cortex, or doses of 0.3-1.0 mg./kg. intravenously, caused replacement of the recurrent IPSP of pyramidal tract cells by a depolarization. Despite this 'reversal' of the IPSP, these neurones failed to fire during the period corresponding initially to the recurrent inhibition. Local anaesthetic-like actions of these high concentrations of strychnine were also noted, as the spikes were reduced in size to a greater extent than would correspond to the change in membrane resting potential.

Since it is possible that strychnine blocks post-synaptic inhibition in the spinal cord by preventing access

of the inhibitory transmitter to the receptor sites (Bradley, Easton and Eccles, 1953; see also Curtis, 1963), it may be proposed that the transmitter(s) responsible for the long-duration IPSP's of cerebral, cerebellar, thalamic and hippocampal neurones (cf. Andersen et al., 1963), and the similarly long-lasting inhibition of olfactory bulb mitral cells (Green, Mancia and von Baumgarten, 1962; Phillips, Powell and Shepherd, 1963; Shepherd, 1963), all of which are also strychnine-resistant, is different from that involved in postsynaptic inhibition at the spinal cord level. On the other hand, one substance may be the inhibitory transmitter at all sites within the c.n.s., but manifest differences in its behaviour and in its susceptibility to blockade by strychnine because of differences in the configuration of the subsynaptic receptor sites. An example of this modification of action of a single compound by differences in its receptor site is provided by acetylcholine at the neuro-muscular junction and upon the heart. In the one case, ACh causes a depolarization which is competitively blocked by d-tubocurarine (e.g. del Castillo and Katz, 1957) and in the other a prolonged hyperpolarization abolished by atropine (e.g. Burgen and Terroux, 1953).

(ii) Electrophoretic ejection of strychnine

Difficulties have previously been encountered in controlling the diffusional efflux of strychnine from solutions within the glass microelectrodes (e.g. Curtis and Watkins, 1960b; Curtis, 1962). Solutions of 10 mM strychnine hydrochloride in 165 mM NaCl have, however, been shown to be finely controllable by retaining voltages of 0.5 V (see Biscoe and Curtis, 1965).

When ejected with currents of 10-20 nA near a total of seven Purkinje cells, strychnine showed a definite though slowly-developing excitant action, either firing the cell directly, or facilitating excitation by DL-homocysteic acid. Fig. 42 illustrated one such experiment, in which the excitation by strychnine was suppressed during the passage of the (cationic) ejecting current. Larger doses of strychnine manifested an additional, local anaesthetic-like, action. In the cell of Fig. 42, 60 nA of strychnine initially fired the cell with a very broad, small spike which eventually disappeared (downward arrow). The spike reappeared (upward arrow) some 10-12 seconds after the end of the strychnine ejection, and gradually increased to its normal size over a further period of about ten seconds. Residual excitation by this dose of strychnine was shown by a persistent increase in discharge rate of the cell for some two minutes.

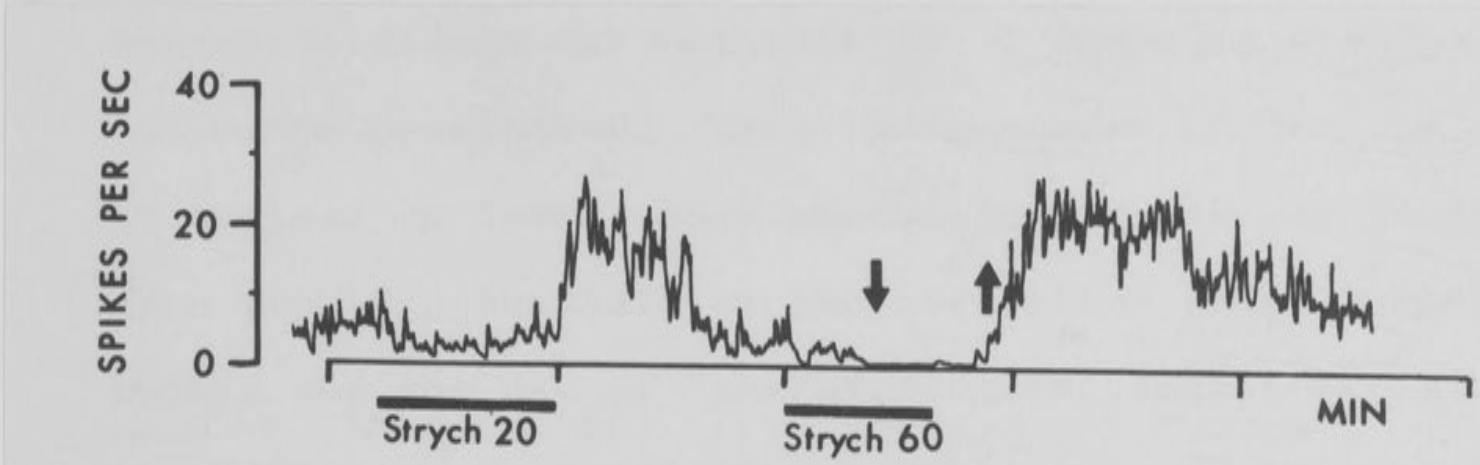

Fig. 42: Effects of electrophoretic ejection of strychnine upon the same Purkinje cell as in Fig. 38. Throughout the period shown, a small amount of DLH was ejected to maintain a "background" firing rate (current = $2\frac{1}{2}$ nA). Strychnine was ejected with cationic currents of 20 and 60 nA at the times shown by the bars beneath the frequency record. At the downward arrow, the spikes of this cell became much smaller and eventually vanished. Spikes reappeared at the upward arrow following the termination of the strychnine administration.

Further description in text.

Ordinate: Firing frequency (spikes/second).

Abscissa: Time scale in minutes.

The high-frequency excitation of cerebellar cells following topical strychnine (e.g. Dow and Moruzzi, 1958, pages 178-9) may be due in part to this direct excitant action, although the possibility of indirect actions cannot be overlooked. This is true also of the effects of topical or intravenous administration of strychnine upon evoked potentials in the cerebellum (e.g. Bremer, 1958), and the use of 'pharmacological tools' of this type led Purpura and Grundfest and their collaborators into the belief that the cerebellar cortex was 'poorly endowed with inhibitory synapses' (e.g. Purpura and Grundfest, 1957; but cf. Purpura, 1959, page 144).

Strychnine has not been tested by electrophoretic ejection near Betz cells.

(b) Ergothioneine

This substance has recently been identified as the 'cerebellar excitatory factor' (Crossland, Woodruff and Mitchell, 1964) previously found in cerebellar extracts (Crossland and Mitchell, 1956). However, when ejected electrophoretically as a cation from a 0.1 M solution of pH 3, this substance neither excited nor depressed the ten Purkinje and seven unidentified cells at 0.38-0.6 mm. depth (presumed to be granular layer cells) upon which it was tried. Similar negative findings have also recently

been reported by Krnjević, Randić and Straughan (1965), and it thus appears unlikely that the increase in the electrical activity of the cerebellum which follows intra-arterial injection of ergothioneine (see Crossland et al., 1964) can be due to a direct action on cerebellar neurones.

(c) 5-Hydroxytryptamine

Upon 19 of 29 pyramidal cells and 8 out of 12 unidentified cerebral cortical neurones 5-hydroxytryptamine (5HT; ejected with currents of 20 to 100 nA) was found to depress both the spontaneous and amino acid induced firing to a greater degree than did corresponding currents carrying Na^+ ions. This depression by 5HT was exerted on ACh-sensitive and insensitive cells alike, and closely resembled the depression of ventrobasal thalamic neurones described by Andersen and Curtis (1964b). Characteristically, the latency of onset and offset was short (1-2 seconds), and the depression was distinct from the interaction demonstrated for 5HT and the synaptic transmitter at the lateral geniculate relay (Curtis and Davis, 1962).

Fig. 43 illustrates the action of 5HT upon a Betz cell with a 4 msec. latency to pyramidal stimulation. Initially, a somewhat irregular excitation was produced by the ejection of DLH with a current of 4 nA. A cationic current of 22 nA through a saturated solution of 5HT-

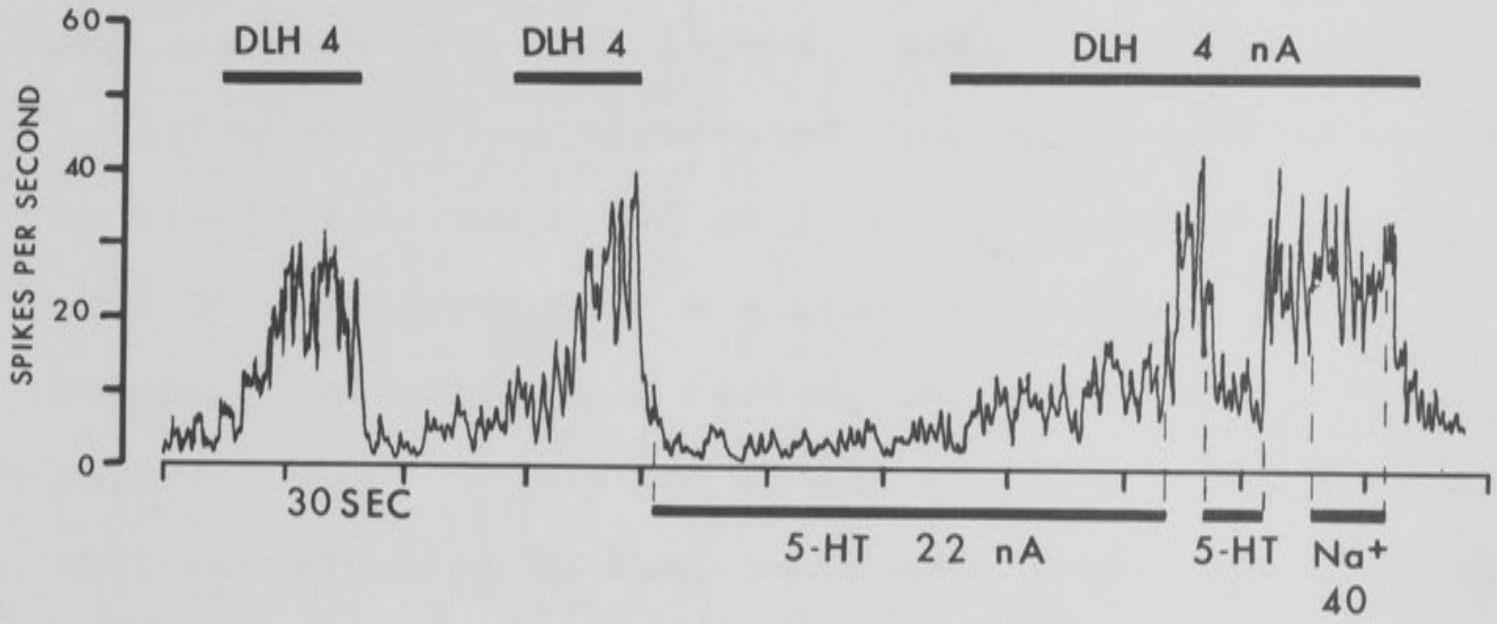

Fig. 43: Responses of a Betz cell to DL-homocysteic acid (DLH) ejected with currents of 4 nA, and the influence of concurrent ejection of 5-hydroxytryptamine (5HT, 22 nA) and of a cationic current carrying sodium ions (Na^+, 40 nA).

Further description in text.

Ordinate: Firing frequency (spikes/second).

Abscissa: Time in minutes.

creatinine sulphate (5HTCS) was then passed for just over two minutes. During this time there was little apparent effect on the low spontaneous firing rate of the cell, but the effectiveness of concurrently-ejected DLH was much less than before. Almost immediately the ejection of 5HT was terminated, the amino acid continuing to be ejected, there was an increase in firing rate to over 30 spikes/second. A second brief ejection of 5HT abruptly curtailed this excitation, and again recovery was very swift at the end of its ejection. To determine what contribution to this depressant effect the cationic current itself may have made, sodium ions were then passed with a current of 40 nA from an adjacent barrel without producing a comparable depression of the amino acid induced firing rate. Inasmuch as the current through the 5HT barrel will also have been conveyed in part by the creatinine of the 5HTCS complex, the potency of 5HT as a depressant must be ranked at least 3-4 times that of the current in this comparison.

Essentially similar results were obtained in an experiment on neurones in the visual (striate) cortex, in which all seven cells tested were found to be depressed by 5HT, using currents of 10-30 nA.

(d) 3-Hydroxytyramine (Dopamine)

This compound, like 5HT, depressed the amino acid firing of the pyramidal and unidentified neurones upon which it was ejected.

(e) Picrotoxin and Pentamethylenetetrazol

When injected systemically in doses of 0.9-1.1 mg./kg. (picrotoxin) and 80-90 mg./kg. (metrazol), these compounds had no effect upon the relationship between firing frequency and the duration of the pause in amino acid induced firing of Betz and Purkinje cells following a pyramidal tetanus and local surface stimulation respectively. Since both recurrent inhibition and basket-cell inhibition are postsynaptic, the failure of picrotoxin to affect the duration of the pause is perhaps not surprising as Eccles, Schmidt and Willis (1963) have shown this compound to have no effect on postsynaptic inhibition in the spinal cord.

SECTION X - ANAESTHETICS

(a) Introduction

The mechanism of action of anaesthetics on nerve cells has been extensively reviewed (for example, Shanes, 1958; Paton and Speden, 1965), and there have been recent reports that the pharmacological behaviour of spinal neurones is modified by anaesthetic agents, including pentobarbitone (Marley and Vane, 1963) and chloralose (Haase and van der Meulen, 1961; Biscoe and Krnjević, 1963).

As the majority of studies of the pharmacology of the cerebral cortex have been made in anaesthetized preparations (Krnjević and Phillis, 1961, 1963a-d; Spehlmann, 1963; Crawford and Curtis, 1964) it was of importance to determine whether the results hitherto presented have been significantly altered by anaesthesia. For example, the large variation between cats in the proportion of ACh-sensitive cortical neurones has been attributed to factors including the depth of anaesthesia (Krnjević and Phillis, 1963a), and the output of acetylcholine from the cerebral cortex has been shown to be specifically reduced by chloralose (Mitchell, 1963).

The depression of synaptic transmission by anaesthesia (e.g. Larrabee and Posternak, 1952; Somjen and Gill, 1963; Somjen, 1963; Løyning, Oshima and Yokota, 1964) may result in several ways, including:-

(i) Presynaptic anaesthetic actions which reduce transmitter release either by depressing its synthesis, interfering with the mobilization of stored transmitter, or by effects on the mechanism of release by presynaptic impulses. For a review of the processes involved in transmitter release, upon any of which the anaesthetic may act, see Eccles (1964, chap. VI).

(ii) Intrasynaptic actions affecting the amount of transmitter which reaches the receptor sites. Possibilities include the formation of inactive complexes, or the activation by the anaesthetic of enzymes which destroy the transmitter.

(iii) Actions upon the postsynaptic cell. The anaesthetic may actually occupy the receptor site to prevent access of the transmitter. Alternatively, the anaesthetic may reduce the conductance changes involving particular ions (for instance, Maeno and Edwards (1965) have found procaine specifically to interfere with sodium conductance in the generation of the end-plate potential of the frog neuromuscular junction). Both the above mechanisms will reduce the size of the postsynaptic

potential. Yet again, the anaesthetic may 'stabilize' non-specialized postsynaptic membrane (cf. Shanes, 1958), or uncouple the alterations in sodium ion conductance involved in spike generation from the membrane depolarization which normally triggers them. This is the mechanism proposed by Schoepfle (1957) and by Shanes, Freygang, Grundfest and Amatniek (1959) to account for the actions of local anaesthetics on axonal conduction without alteration of resting membrane potential and resistance.

Essentially similar mechanisms to those of types (ii) and (iii) above can be postulated to account for the decreased sensitivity of a cell to locally-ejected excitants in the presence of anaesthetics, whether the excitants are proposed to act upon synaptic receptors or at other sites on the postsynaptic cell membrane.

The experiments in this Section were performed on unanaesthetized cerveau isolé cats, unless otherwise stated. Acetylcholine-sensitive cells of the pericruciate cerebral cortex were observed as described below over control periods of at least 5-10 minutes before any anaesthetic was given in order to ensure stability of the recording conditions and of the responses. The femoral arterial blood pressure was continuously monitored during the 'run', and the amplitude and shape of the extracellular spikes

carefully monitored on the oscilloscope screen in order to detect any relative movement of the cell with respect to the microelectrode tip. Such movements were particularly troublesome whenever administration of the anaesthetic produced marked changes in the animal's blood pressure or pattern of respiration. Drugs were given as slowly as practicable, and many of the animals had previously been paralysed and artificially respired in an attempt to reduce these artefacts. Results from experiments in which the extracellular spike shape changed concurrently with the systemic blood pressure were disregarded.

Alternate ejections of acetylcholine and DL- homocysteic acid were used as a means of testing chemical sensitivity of single cortical neurones. Doses of the excitants were chosen to produce submaximal rates of firing of the cell, and were separated by sufficiently long intervals to enable complete recovery of the 'background' firing to control levels before the next excitant was ejected. If, as was assumed, ACh and DLH act entirely upon the (postsynaptic) cell whose spikes were being recorded, no information can be gained about presynaptic actions or specific interference with the transmitter by the anaesthetic agents used, except insofar as the spontaneous 'background' firing is modified. In particular, depression of transmitter release (cf.

Mitchell, 1963; Løyning, Oshima and Yokota, 1964; Paton and Speden, 1965) will not be revealed.

(b) Effects of intravenous barbiturates

(i) Pentobarbitone sodium (Nembutal, Abbott), was used as the basal anaesthetic in most of the preliminary experiments, and was also the most thoroughly investigated of the barbiturates in this series. In the unanaesthetized cerveau isolé preparations of Fig. 44, 10 mg./kg. of pentobarbitone was injected intravenously at the arrow, and resulted in a rapid decline of sensitivity of the cell to both acetylcholine (100 nA) and DL-homocysteic acid (13 nA). There was no associated change of spike shape or size, nor was the fall in chemical sensitivity concurrent with the slight fall in mean blood pressure which followed the injection of pentobarbitone. Whereas the maximal effect on the blood pressure occurred about half a minute after the end of injection, and recovery occurred gradually thereafter, maximal depression of both acetylcholine and DLH responses was delayed 7-10 minutes and recovery was incomplete after some 50 minutes. It is noteworthy that both the depression and the recovery of chemical sensitivity occurred with the same time-course for both excitants. This finding, and the time-course illustrated in Fig. 44, were characteristic of

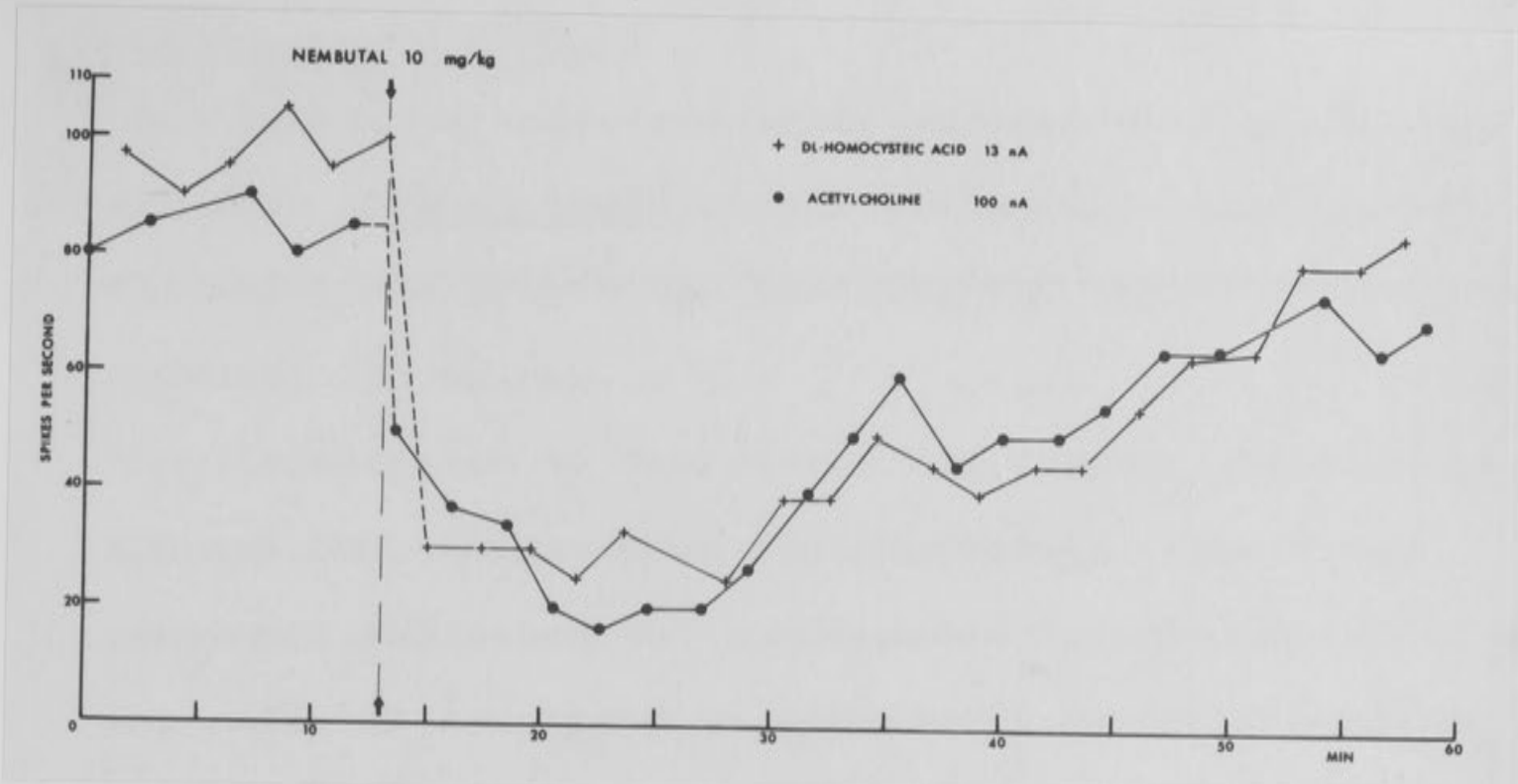

Fig. 44: In this and the following five figures, the peak firing frequencies of pericruciate cortical neurones evoked by alternate electrophoretic ejection of DL-homocysteic acid and acetylcholine are plotted. All cats were unanaesthetized cerveau isolé animals. In each case, care was taken to ensure that the responses to each excitant were submaximal, and one or more doses for an anaesthetic agent were then given.

This figure shows the effect of intravenous pentobarbitone (NEMBUTAL, 10 mg/kg) upon the firing evoked by DLH (13 nA, +) and ACh (100 nA, ●). Further description in text.

Ordinate: Peak firing frequency during each ejection.

Abscissa: Time in minutes.

pentobarbitone injection in doses from 2-10 mg./kg. in all five cerveau isolé preparations tried, and in one cat which had previously been lightly anaesthetized with nembutal (35 mg./kg.).

In addition to decreasing the responses evoked by ACh and DLH, intravenous pentobarbitone reduced and sometimes abolished the spontaneous background firing of the cell, or converted an apparently random discharge into 'spindles' (Dempsey and Morison, 1943; Andersen and Curtis, 1964a). It may be proposed that the observed reduction in chemical responsiveness after the administration of the anaesthesia was due to the removal of this background synaptic barrage which had previously held the cell in a partially depolarized state. However, when small amounts of DLH were ejected continuously in an attempt to restore this 'background firing', superimposed doses of ACh or DLH failed to evoke responses of the same size as the controls, and on occasion the addition of these test doses of the excitants merely led to the rapid development of a 'depolarization block' (Section III (b) above).

Furthermore, if the background synaptic barrage had made a large contribution to the total depolarization of the cell in the presence of the chemical excitants, its removal ought to depress low-frequency firing (in response

to just-suprathreshold doses of ACh or DLH) more than high-frequency excitation. However, anaesthesia did not seem to reduce low rates of chemical excitation to any greater extent than it did intermediate frequencies. Thus, the removal of the normal background depolarization by anaesthesia is not the only mechanism whereby the efficacy of chemical excitation is reduced. On the contrary, the decline in spontaneous firing of the cell after administration of a general anaesthetic appears rather to reflect decreased postsynaptic sensitivity to all chemical excitants, the synaptic transmitter(s) included.

(ii) Thiogenal (Sodium methylthioethyl-2-pentyl-thiobarbiturate; Merck AG) was administered intravenously in doses of 5-20 mg./kg. to five cats. In each case, a virtually parallel reduction in chemical sensitivity to both ACh and DLH was observed, as a rule reaching its maximum depth between 2 and 5 minutes after the injection. As with pentobarbitone, these effects were not associated with apparent changes in the cell spike (i.e. with relative movement of the cell and the electrode tip). The time-course of depression and recovery of chemical responsiveness was shorter after thiogenal than after nembutal, however, and somewhat more closely resembled that of the fall in mean femoral blood pressure.

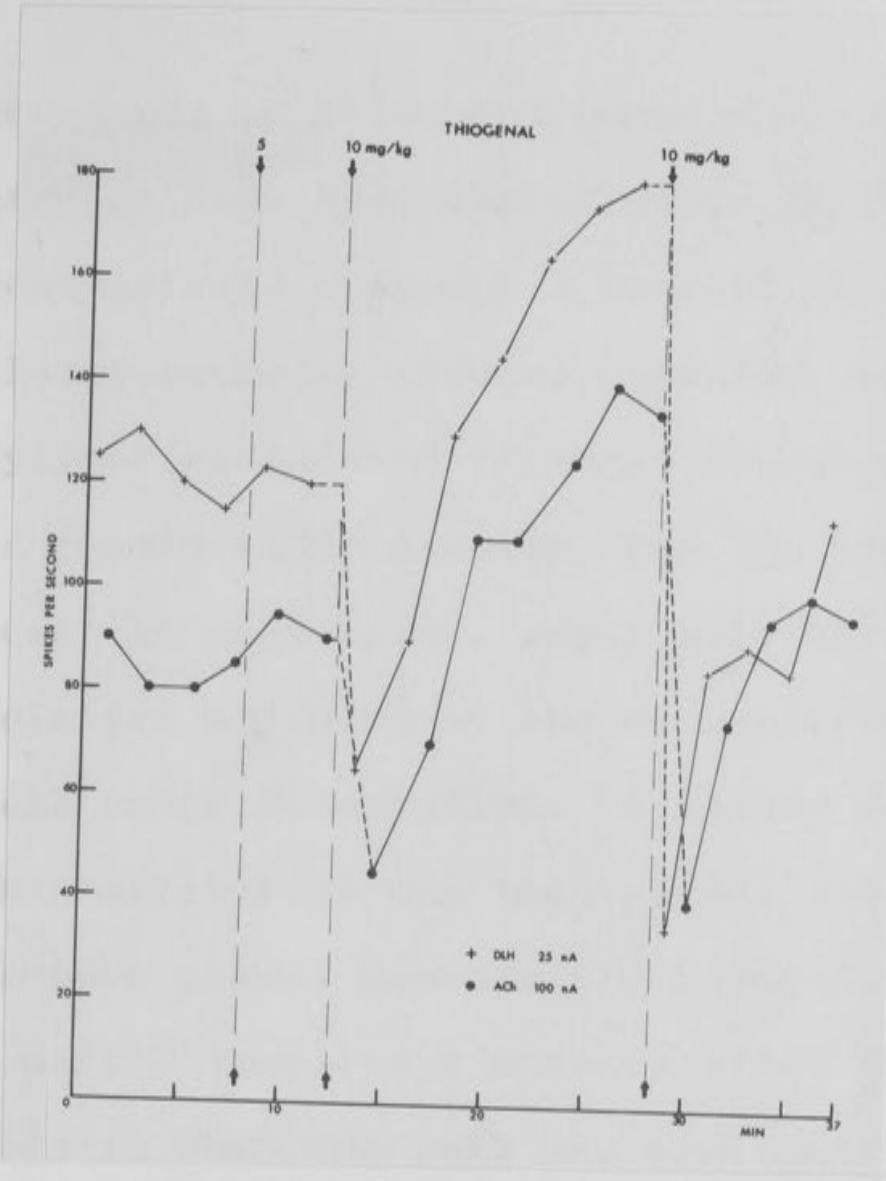

Fig. 45: Effects of three doses (5, 10 and 10 mg/kg) of Thiogenal given intravenously upon the responses of a pericruciate neurone to DLH (25 nA, +) and ACh (100 nA, ●). Further description in text.

Ordinate: Firing frequency in spikes/second.

Abscissa: Time in minutes.

Three doses of thiogenal were given in the experiment illustrated in Fig. 45. The first (5 mg./kg.) produced no apparent effect, although a dose of 10 mg./kg. $4\frac{1}{2}$ minutes later markedly reduced both the amino acid and the acetylcholine-induced firing. Recovery occurred to responses considerably greater than the controls (some 150 per cent in each case), apparently because of some minute relative movement of the microelectrode with respect to the cell under observation. A second dose of 10 mg./kg. of the thiobarbiturate was then given, and again both responses were almost immediately reduced, but recovery was only partly complete 9 minutes after the second dose of anaesthetic when the cell was accidently impaled by the electrode.

(iii) Diallylbarbituric acid (Dial, Ciba Ltd.) was given intravenously in two doses, each of 5 mg./kg., to one cat and each time caused decreased spontaneous activity and a fall in sensitivity to acetylcholine without much change in the DLH response of one neurone. In other cats, however, no such specific action against ACh-sensitivity could be demonstrated, and in a series of 3 cats anaesthetized with Dial (35-40 mg./kg.) or Dial compound (Ciba Ltd., Dial 100 mg./cc. plus Urethane 400 mg./cc., 0.4 cc./kg.), every Betz cell identified could be readily

excited by ejection of acetylcholine, and the overall proportion of ACh-sensitive pericruciate neurones was the same as in animals anaesthetized with nembutal.

(c) Other systemic anaesthetic agents

(i) α-Chloralose (British Drug Houses Ltd.; Fluka AG) was freshly dissolved in hot water, allowed to cool, filtered and injected in doses of 30-50 mg./kg. intravenously during the observation of cells in two preparations, one of which is illustrated in Fig. 45. Over a period of 3-5 minutes, the responses to both acetylcholine and DL-homocysteic acid and the spontaneous background firing of the cells were abolished. The cells were observed for up to 30 minutes without any evidence of returning chemical sensitivity. Very large doses of DLH (up to 30-40 nA) would still excite the cells, but it proved impossible to fire them with acetylcholine ejected with currents of 200 nA.

In both cats thus anaesthetized with chloralose, subsequent tracks made with the microelectrode yielded a smaller proportion of acetylcholine-sensitive pericruciate neurones than had been found earlier in the experiment when the animals were unanaesthetized but too few cells were found for statistical significance.

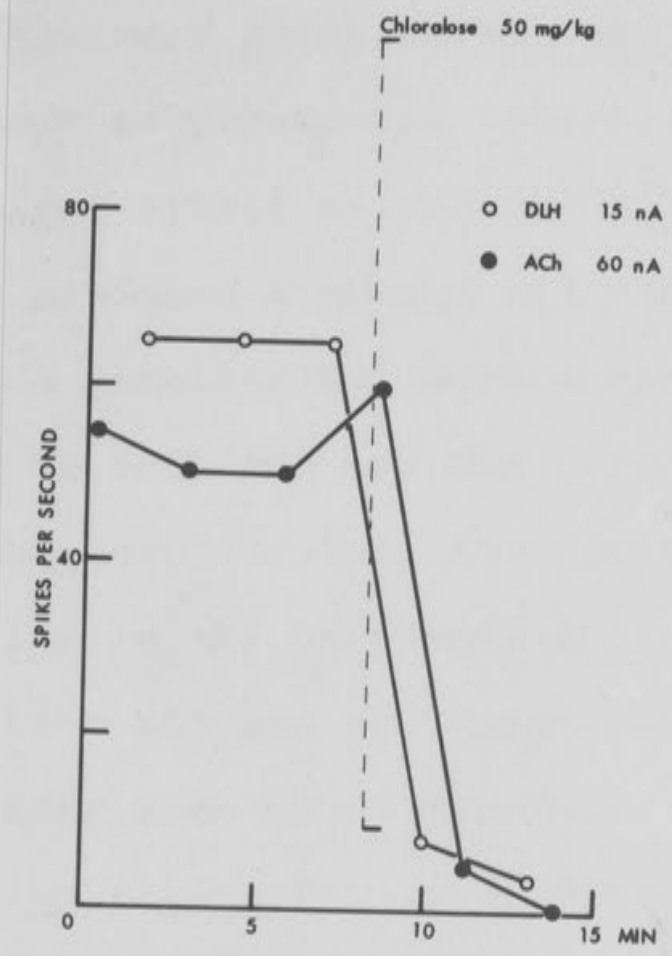

Fig. 46: Effect of a clinically effective anaesthetic dose (50 mg/kg, i.v.) of Chloralose upon the responses of a pericruciate neurone to DLH (15 nA, +) and ACh (60 nA, ●). Further description in text.
Ordinate: Firing frequency in spikes/second.
Abscissa: Time in minutes.

(ii) Urethane (British Drug Houses Ltd.). Doses of 50-200 mg./kg. were given during the observation of four cells in three preparations. Whereas the smaller doses had no apparent effect on the chemical sensitivity, 200 mg./kg. produced a slight reduction in the background firing, and a parallel but more marked decrease in sensitivity to both ACh and DLH (Fig. 47). As was the case with the barbiturates, the diminution and recovery of sensitivity to the excitants followed a similar time-course for both ACh and the amino acid, recovery after urethane taking some 40-60 minutes.

(iii) Halothane (1,1,1-trifluoro-2-bromo-2-chloroethane; Fluothane, I.C.I., Ltd.) was administered as $1\frac{1}{2}$-$2\frac{1}{2}$ v/v. in air through a Palmer pump to paralysed artificially ventilated cerveau isolé cats. In all, seven such experiments were performed on five cats, and in no case was there any differential effect upon the amino acid and acetylcholine sensitivity of the cells. However, because of a marked hypotensive effect of halothane anaesthesia, it was usually difficult to 'hold' the cell in its relationship to the recording microelectrode. In the experiment illustrated in Fig. 48, the mean systolic femoral blood pressure fell progressively over three minutes by 20 mm. Hg., thereafter fluctuating between 95 and 100 mm. Hg., and recovering slowly over some

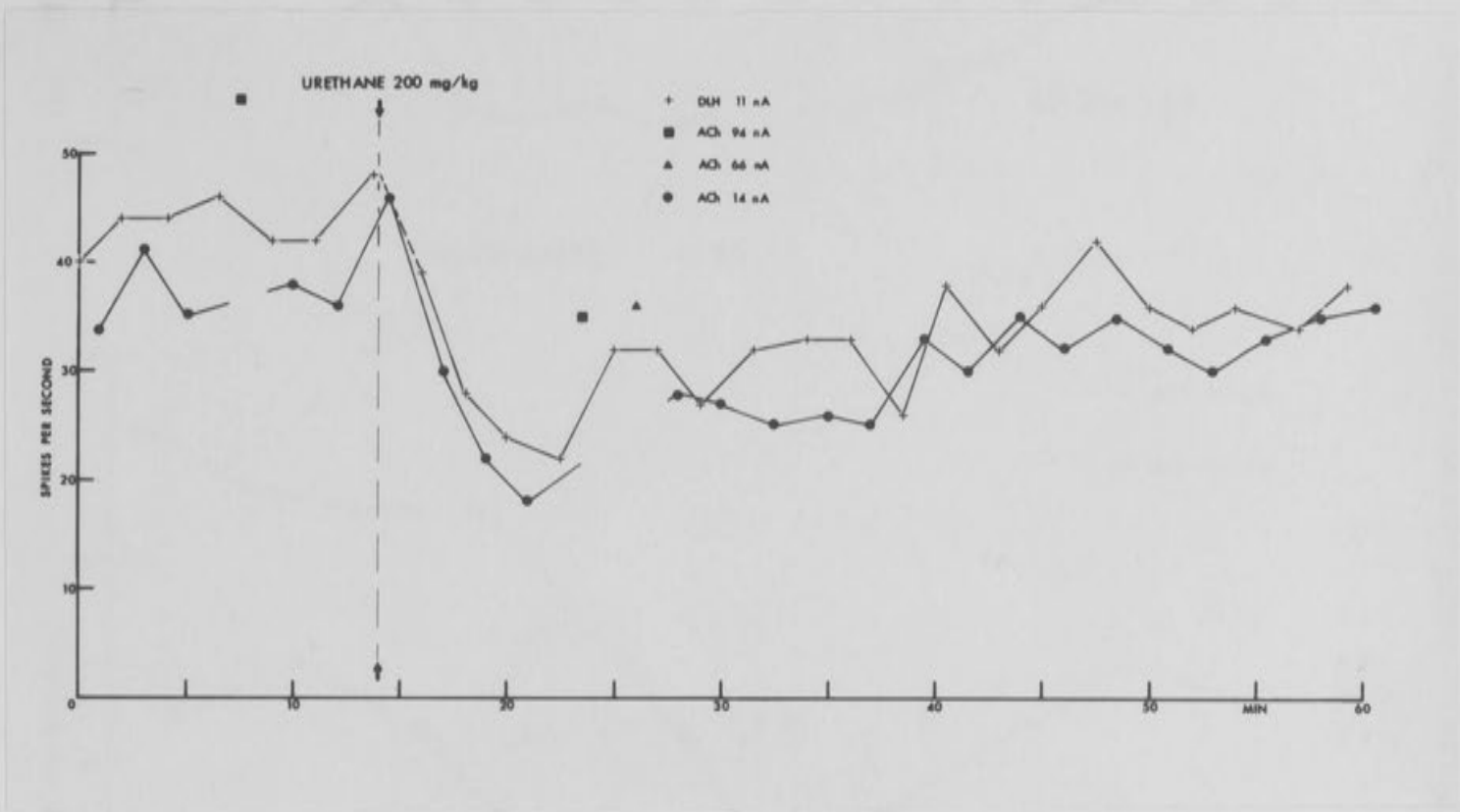

Fig. 47: Effect of intravenous urethane (200 mg/kg) upon the responses of a pericruciate neurone to DLH (11 nA, +) and ACh (14 nA, ●). This cell was unusually sensitive to acetylcholine, apparently maximal responses being evoked by ACh ejected with 66 nA (▲) and 94 nA (■). Ordinate: Firing frequency in spikes/second. Abscissa: Time in minutes.

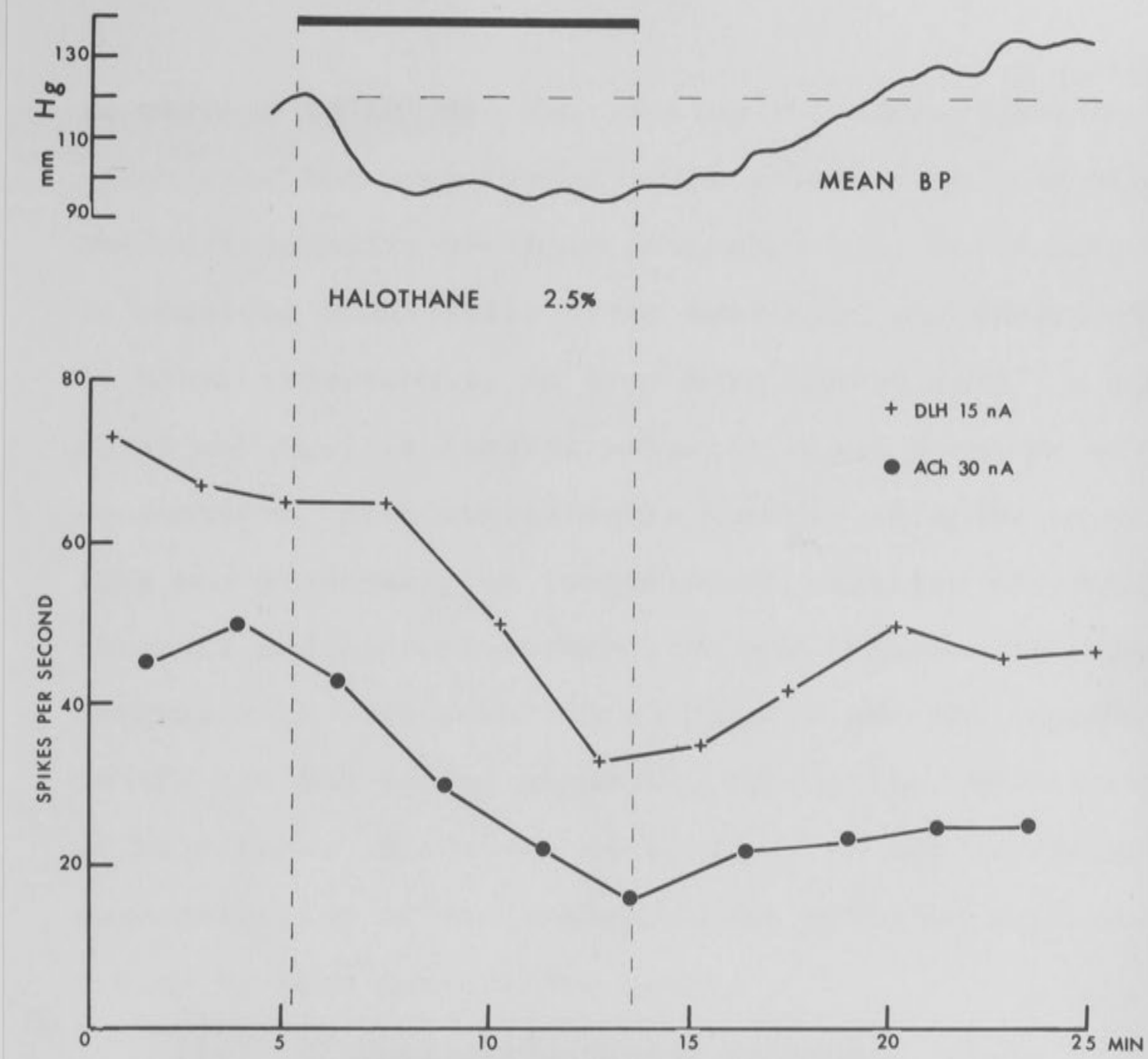

Fig. 48: Effect of 2.5% halothane vapour in air upon the mean femoral arterial bloodpressure (uppermost curve) and the responses of a pericruciate neurone to DLH (15 nA, +) and to acetylcholine (30 nA, ●). The period during which the cat breathed the anaesthetic vapour is shown by the black horizontal bar and the interrupted vertical lines.

Ordinates: Upper curve - mean blood pressure in min. Hg.

Lower curve - firing frequency in spikes per second.

Abscissa: Time in minutes.

12 minutes to 125 mm. Hg. During the administration of halothane, the peak firing rates produced by both DLH and acetylcholine declined progressively, but recovery of chemical sensitivity after halothane was incomplete. In other experiments, an even more marked fall (as much as 45 mm. Hg.) in femoral arterial blood pressure was encountered, with considerable changes in spike size. This was presumably an indication of relative movement of the cell and microelectrode - on one occasion the spike increased in size from 600 to 750 μV, and the responses to ACh and DLH became augmented during the administration of halothane. There was no evidence of any difference in susceptibility of the acetylcholine or amino acid induced diring to this anaesthetic agent.

(iv) Nitrous oxide/oxygen mixtures. These were used in proportions from 60 to 90 per cent nitrous oxide, and produced merely effects attributable to hypoxia when the proportion of oxygen was less than 20 per cent. In the cell illustrated in Fig. 49, for instance, there was no effect of the nitrous oxide mixtures upon either acetylcholine or DL-homocysteic acid sensitivity, although the arterial blood pressure rose and irregular bursts of activity of up to 40/second became prevalent in the 'background' firing during this period. Similar behaviour has

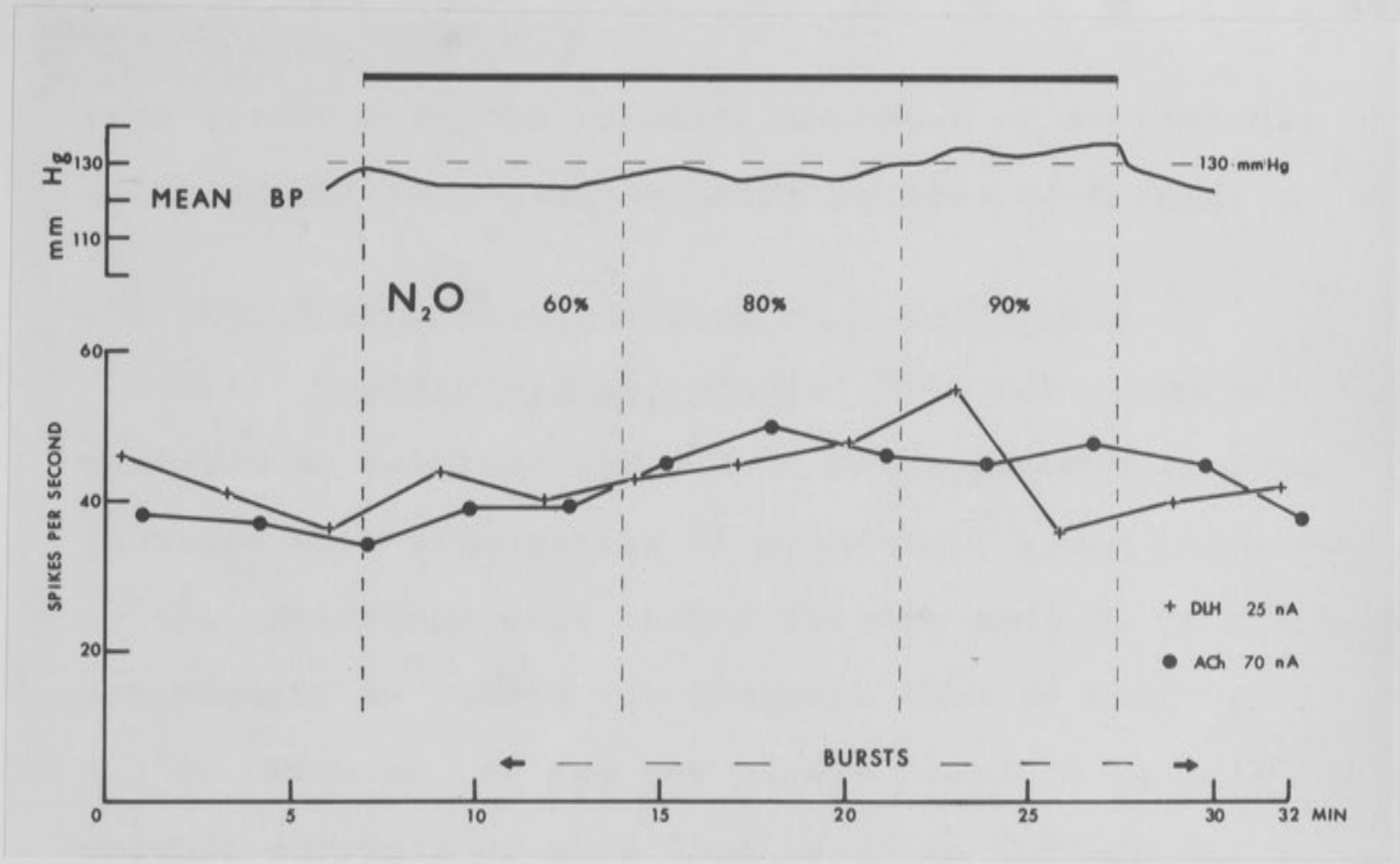

Fig. 49: Effects of various concentrations of nitrous oxide in oxygen in the mixture breathed by an artificially-ventilated cerveau isole cat. The mean femoral arterial blood pressure is shown by the uppermost curve in relation to an arbitrary datum line at 130 mm Hg. The lower graph represents the responses to DLH (25 nA, +) and ACh (70 nA, ●). The concentration of nitrous oxide in the mixture was increased from 60 to 80 and then 90% at the broken vertical lines, after which the animal was returned to room air. During the period indicated, high frequency "bursts" of firing of the cortical neurone was noted.

Ordinates: Upper curve - mean blood pressure in mm. Hg.

Lower curve - firing frequency in spikes per second.

Abscissa: Time in minutes.

been observed in the cortical neurones of another cat deliberately subjected to short periods of anoxia.

(d) Electrophoretic ejection of barbiturates

(i) Pentobarbitone sodium. This substance is unstable in solution (pH 9.3 to 9.6), undergoing ring cleavage with elemination of a carbonyl group (Asplund, 1955). Solutions were either freshly made up before each experiment, or stored for not more than 18 hours at 3-4°C. Even so, it was not always possible to pass anionic currents of more than 20-30 nA through 0.2 M solutions in an electrode barrel, and these barrels often drastically changed their resistance during the ejection of the barbiturate, passage of the current causing so much electrical noise that the cell spike was obscured. Ejection periods for pentobarbitone exceeding 2-5 minutes duration were particularly prone to this trouble, and were avoided wherever possible.

A total of fifteen experiments on eleven pericruciate neurones was performed in cerveau isolé cats, the currents used to eject the barbiturate being between 10 and 100 nA. In Fig. 50, the ejection of pentobarbitone with a current of 20 nA slowed the onset and decreased the maximal rate of firing achieved by DL-homocysteic acid (11 nA), without affecting the firing pattern during ejection of

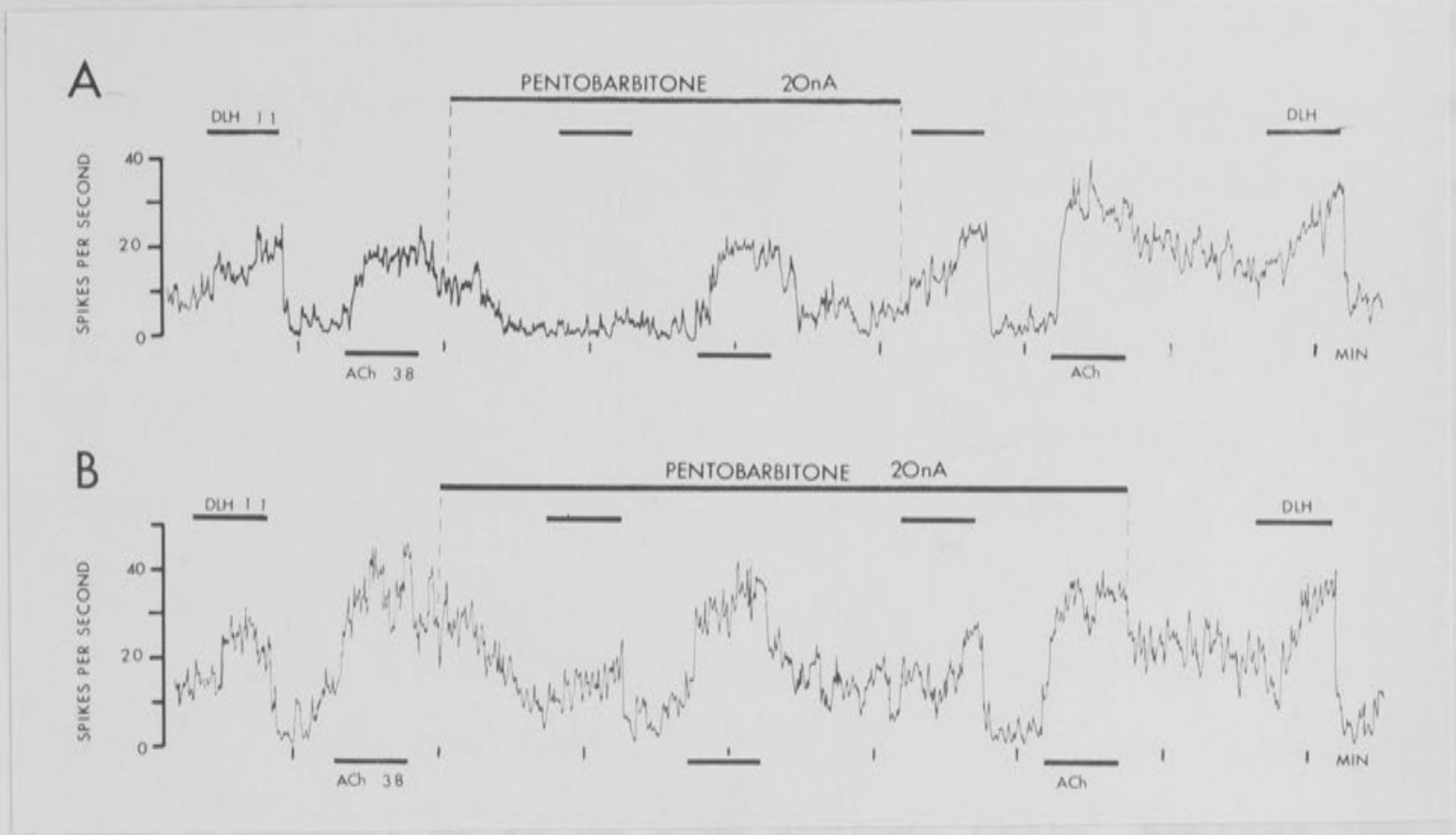

Fig. 50: Effects of electrophoretically ejected pentobarbitone on the responses of a pericruciate neurone in a cerveau izole cat ejection of DLH (11 nA, upper markers) and ACh (38 nA, lower markers). This cell was spontaneously active, and the traces "A" and "B" were taken from separate portions of the same experiment. During the periods shown by the topmost horizontal bars, pentobarbitone was ejected as an anion by currents of 20 nA.

Ordinate: Firing frequency in spikes per second.

Abscissa: Time in minutes.

acetylcholine (40 nA). In the presence of the barbiturate there was rather less background firing, and the prolonged 'tail' of excitation after ACh was also less marked. The sensitivity of this cell to DLH recovered very rapidly after cessation of the barbiturate ejection, but in most of the trials both the spontaneous and amino acid induced firing recovered to control levels within 15-30 seconds after pentobarbitone.

Fig. 51 illustrates essentially similar results obtained during a 460-second ejection of pentobarbitone with a current of 20 nA upon another cell. Whereas the spontaneous activity and DLH-responses were virtually abolished, the peak firing frequency produced by a submaximal dose (30 nA) of acetylcholine declined much more slowly and to a lesser extent. Recovery was again rapid after the end of the barbiturate ejection. In this figure, the mean level of spontaneous firing over 5-10 seconds before the ejection of each excitant is plotted, with the vertical bars indicating extreme variations in the height of isolated 'bursts' of activity.

Another estimate of the time-course of action, and of the relative susceptibility of excitation by ACh and by the amino acid to the action of locally ejected pentobarbitone was obtained by studying the effects of the barbiturate upon steady rates of firing produced by

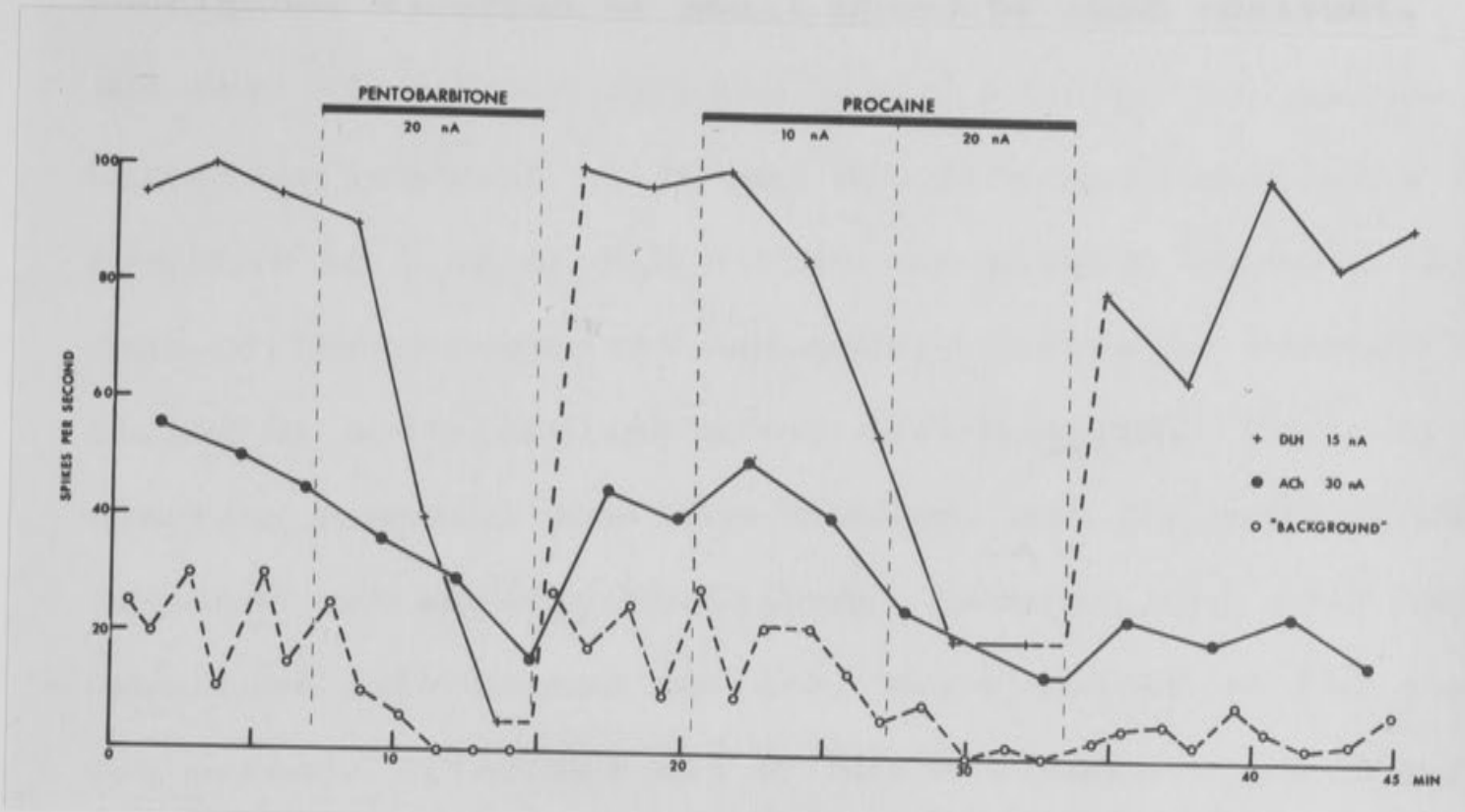

Fig. 51: Effects of electrophoretic pentobarbitone and procaine upon the responses of a pericruciate neurone in a cerveau isolé cat to DLH (15 nA, +) and ACh (30 nA, ●), as well as upon the spontaneous "background" firing (o--o) measured just prior to each dose of the excitants. Pentobarbitone was ejected as an anion with a current of 20 nA during the first signal, and procaine as a cation with currents of 10 and 20 nA during the second. Further description in text.

Ordinate: Firing frequency in spikes per second.

Abscissa: Time in minutes.

continuous ejection of small doses of each excitant. In the cell with the responses traced in Fig. 52, pentobarbitone (current of 30 nA) had virtually abolished the response to 7 nA of DLH within one minute, whereas this dose of the anaesthetic had merely partially reduced the firing by acetylcholine after five minutes. The current ejecting nembutal was then doubled, and the acetylcholine-response was rapidly abolished. However, the cell spike simultaneously became smaller, and remained so for some ten seconds after the end of the anaesthetic administration.

In every case, ejection of pentobarbitone slowed the onset of excitation of these neurones by the amino acid, and reduced the peak frequency evoked by DLH more readily than that produced by acetylcholine. The spontaneous 'background' firing of the cells was also reduced, with a similar time-course to that of the depression of the amino acid sensitivity. Spike changes were usually only evident with large ejecting currents or prolonged administration of pentobarbitone, but this phenomenon was not fully investigated.

(ii) Phenobarbitone sodium. This substance was ejected with currents of 30-60 nA into the vicinity of three cells, but it proved difficult to affect the chemical sensitivity without spike alteration, and in each case

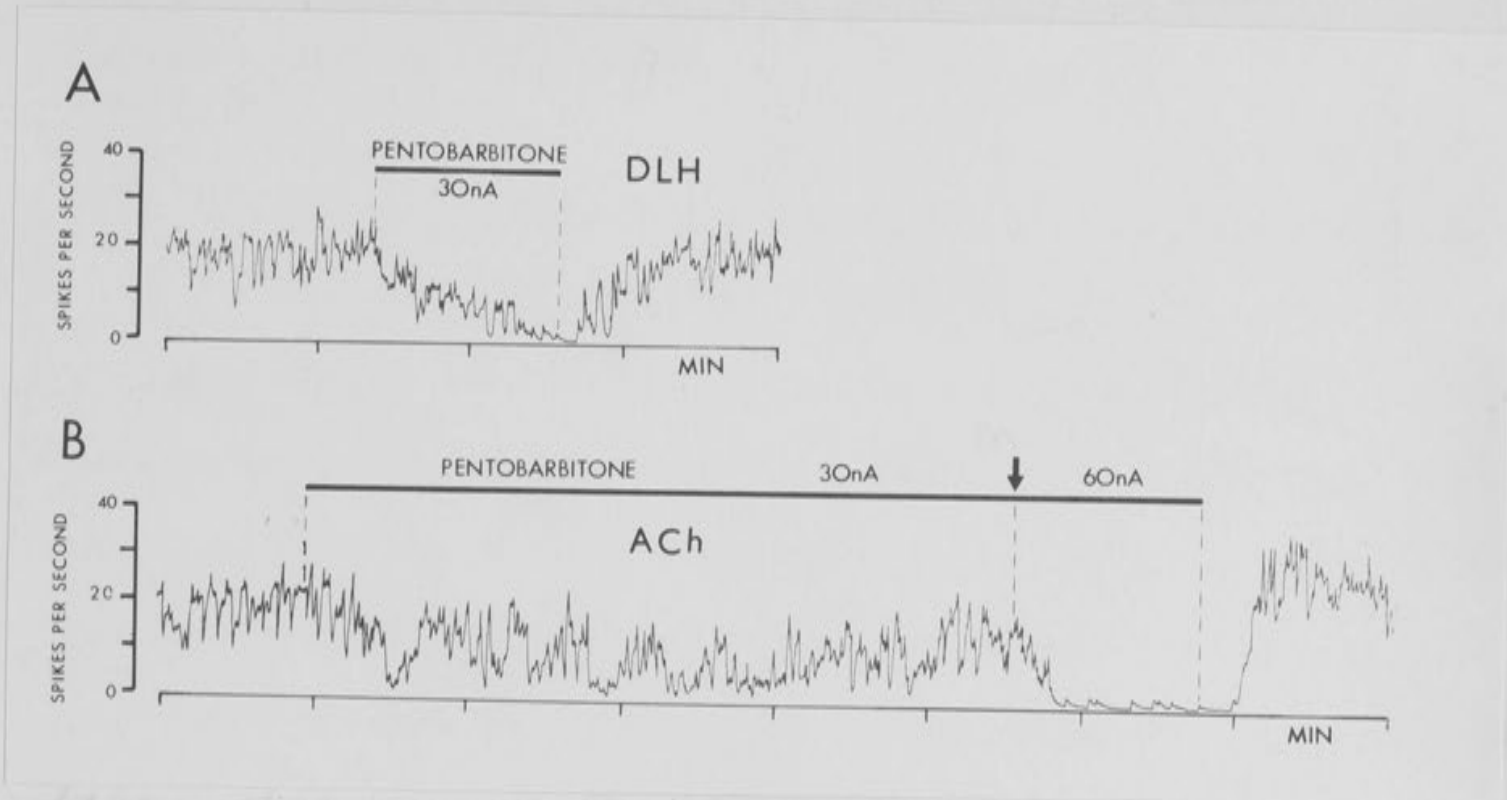

Fig. 52: Effects of electrophoretic ejection of pentobarbitone upon the excitation of a pericruciate neurone in a cerveau isolé cat by DLH (7 nA, on throughout the period shown in "A") and by ACh (current of 40 nA, on throughout the period shown in "B"). These traces were made from successive portions of the original record. Further description in text.

Ordinate: Firing frequency in spikes per second.

Abscissa: Time in minutes.

ejection of phenobarbitone reduced both DL-homocysteic acid and acetylcholine sensitivity of the cell to a similar degree (Fig. 53).

(e) Effects of procaine on cerebral cortical neurones

It was now of interest to establish the effect of a typical 'local anaesthetic' upon cortical neurones, and to determine whether the tendency for locally-ejected anaesthetics to reduce the amino acid induced excitation was due merely to the fact that both substances were in highest concentration upon the same area of cell membrane (i.e., that nearest the electrode tip). For these experiments, procaine was selected as representative of the local anaesthetic agents (see also Curtis and Phillis, 1960), although del Castillo and Katz (1957) had shown it to have a specific antagonism to ACh-excitation at the motor end-plate.

When ejected from an 0.1 M solution of the hydrochloride with currents of 10 and 20 nA (Fig. 51), procaine reduced the sensitivity of this cell to both DL-homocysteic acid and acetylcholine, and progressively decreased the spontaneous firing also. Although the responses to DLH recovered rapidly after the local anaesthetic was removed, the spontaneous activity and acetylcholine-sensitivity had not fully returned 15 minutes afterward,

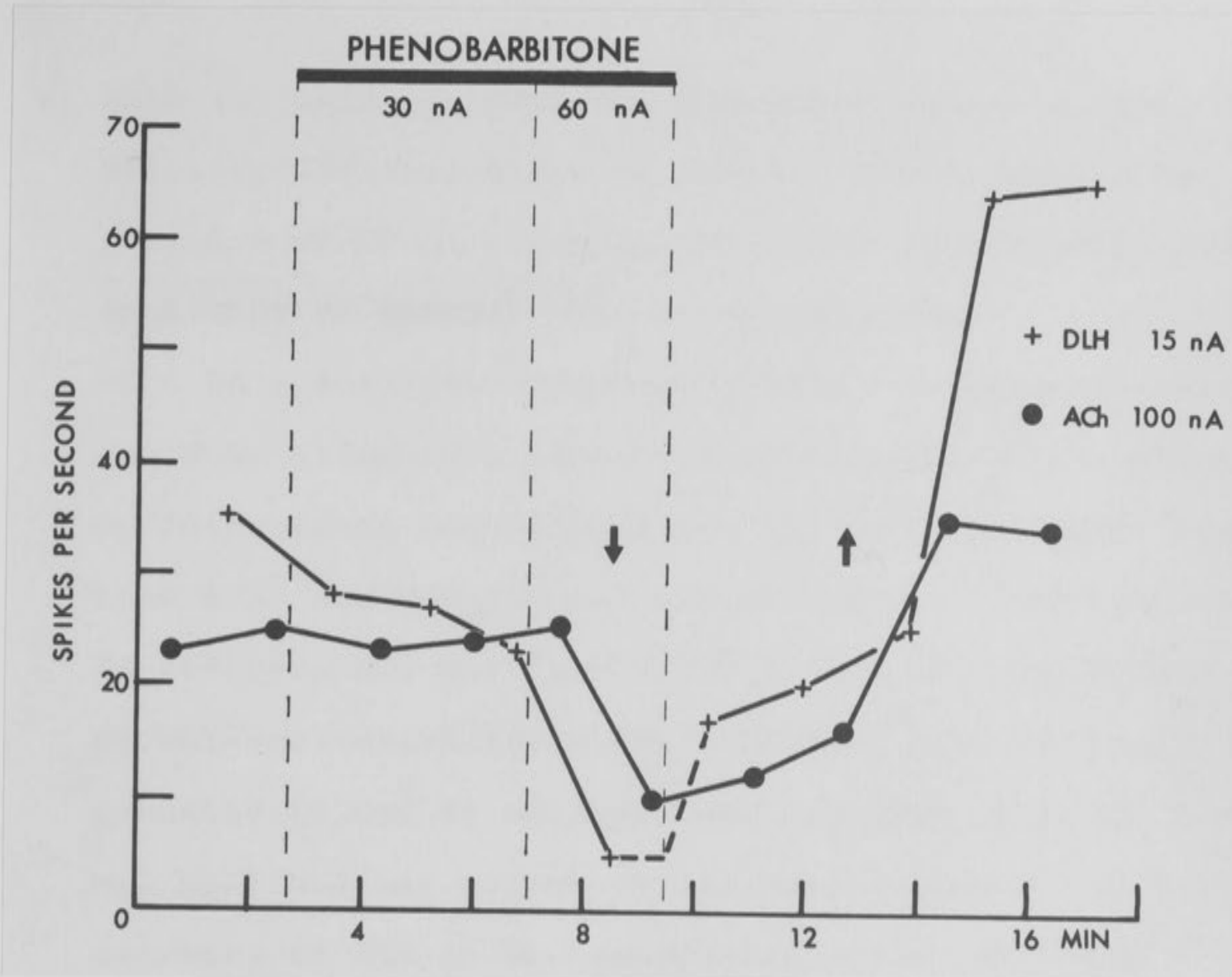

Fig. 53: Effect of electrophoretic ejection of phenobarbitone (currents of 30 and 60 nA, upper bar) on the responses of a pericruciate cortical neurone of a cerveau izole cat. Test doses of DLH (15 nA, +) and ACh (100 nA, ●) were used. At the downward arrow during phenobarbitone ejection, the cell spike was markedly reduced, full recovery of spike size being delayed for over three minutes after the end of phenobarbitone administration (upward arrow). Ordinate: Firing frequency in spikes per second. Abscissa: Time in minutes.

when the cell was impaled. In other respects, the proportional depression of DLH and ACh responses by currents of 20 nA conveying procaine is virtually identical with that of similar amounts of nembutal.

If a suitable concentration of a local anaesthetic could be attained at all portions of the cell membrane by intravenous administration, it was anticipated that both ACh- and DLH-sensitivity of cortical neurones should be reduced, and any 'specific' action against either ACh- or DLH-excitation revealed. However, neither intravenous procaine (4 and 4½ mg./kg.) nor procaine amide (2 and 5 mg./kg.) had any effect on the sensitivity of pericruciate neurones to ACh or DL-homocysteic acid. Although intravenously administered procaine is rapidly hydrolysed by serum esterases (Brodie, Lief and Poet, 1948), procaine amide is resistant to enzymic destruction (Mark et al., 1951; Goodman and Gilman, 1955, p.725) and is a local anaesthetic comparable in potency with procaine itself. However, intravenous procaine amide does not share the central actions of i.v. procaine (Mark et al., 1951) and may not even cross the blood-brain barrier because of the low lipid solubility of the hydrochloride.

(f) Comparison of effects of systemic anaesthetics with experiments upon a peripheral muscarinic receptor (guinea-pig ileum)

Krnjević and Phillis (1963b, p.298) have found diallylbarbituric acid to reduce the responses of cortical neurones to ACh to a greater extent than those to glutamate, and McCance and Phillis (1964a) found fewer ACh-sensitive cerebellar neurones in cats anaesthetized with Dial compound (diallylbarbituric acid plus urethane, Ciba Ltd.) than in pentobarbital-anaesthetized or cerveau isolé animals. It was therefore of interest to test these barbiturates upon another tissue having muscarinic receptors. The guinea pig ileum was selected, as in certain respects it 'provides a valuable simple paradigm of the behaviour of the brain itself' (Paton, 1957). Chloralose was also investigated in this series of experiments, although it appears to exert its specific antagonism towards ACh-excitation in the central nervous system by interfering with the release of ACh from synaptic terminals (Krnjević and Phillis, 1963b, c; Mitchell, 1963; Haase and van der Meulen, 1961; but compare also Biscoe and Krnjević, 1964).

In these experiments, the concentrations of anaesthetic used are expressed as w/v, in order to facilitate comparison with the amounts given systemically

in the cat experiments (above). A concentration of 10^{-5} w/v, for instance, is approximately equivalent to a dose of 10 mg./kg. (10^{-5} w/w) intravenously.

Pieces of ileum 2-3 cm. in length were suspended in oxygenated Tyrode solution in a 10 ml. bath maintained at 30°C. ($\pm$ 1°). Contractions of the longitudinal smooth muscle of the ileum were recorded by means of a pen-writer (Servo/Riter, Texas Instruments, Inc.), actuated from an isotonic lever system (1 gram tension) by a photo-electric transducer. Doses of acetylcholine bromide (5-500 ng./ml. of bath volume), histamine acid phosphate or histamine dihydrochloride (0.05-1 g./ml.), or M/4 KCl solution (0.5 or 1 ml.) were injected at $2\frac{1}{2}$-$3\frac{1}{2}$ minute intervals. After each dose, the bath was drained and flushed twice with the bathing solution.

The anaesthetics used (Nembutal, Dial and Thiogenal, each in concentrations from 10^{-6} w/v to 10^{-4} w/v, and chloralose, 10^{-4} w/v) were dissolved in oxygenated Tyrode solution, and the reservoir which filled the bath was charged with the appropriate solution after the control responses had been obtained. In most experiments recovery (when normal Tyrode was again substituted for that containing the anaesthetic agent) was incomplete.

Some degree of spontaneous contraction was usually evident in the preparations despite the low temperature

of the bath. In plotting the response curves, the height of the peak contraction was measured above this background activity. Particularly with doses of acetylcholine exceeding 50 ng./ml., 'fade' was frequently observed (cf. Paton, 1961), with the result that the mean response was considerably less than the initial peak. This phenomenon was accentuated by the anaesthetic agents tried, even in quite small doses. In some experiments an irregular and irreversible depression of the maximal responses occurred. The specificity of action of the anaesthetics against ACh and histamine or K^+ responses was measured by the dose-ratio (of concentrations required to produce 50 per cent of maximal response in presence of anaesthetic compared with the controls). Dose ratio values for ACh were usually close to those for K^+ or histamine, but occasionally were 3-5 times higher, suggesting a slight preferential effect on the acetylcholine-sensitivity (Fig. 54). In general, however, the pattern observed resembled that of cortical neurones subjected to anaesthetic agents by systemic administration. The slightly greater effect of barbiturates on ACh-responses of the ileum may have been accentuated by the increased 'fade' in the presence of anaesthetics.

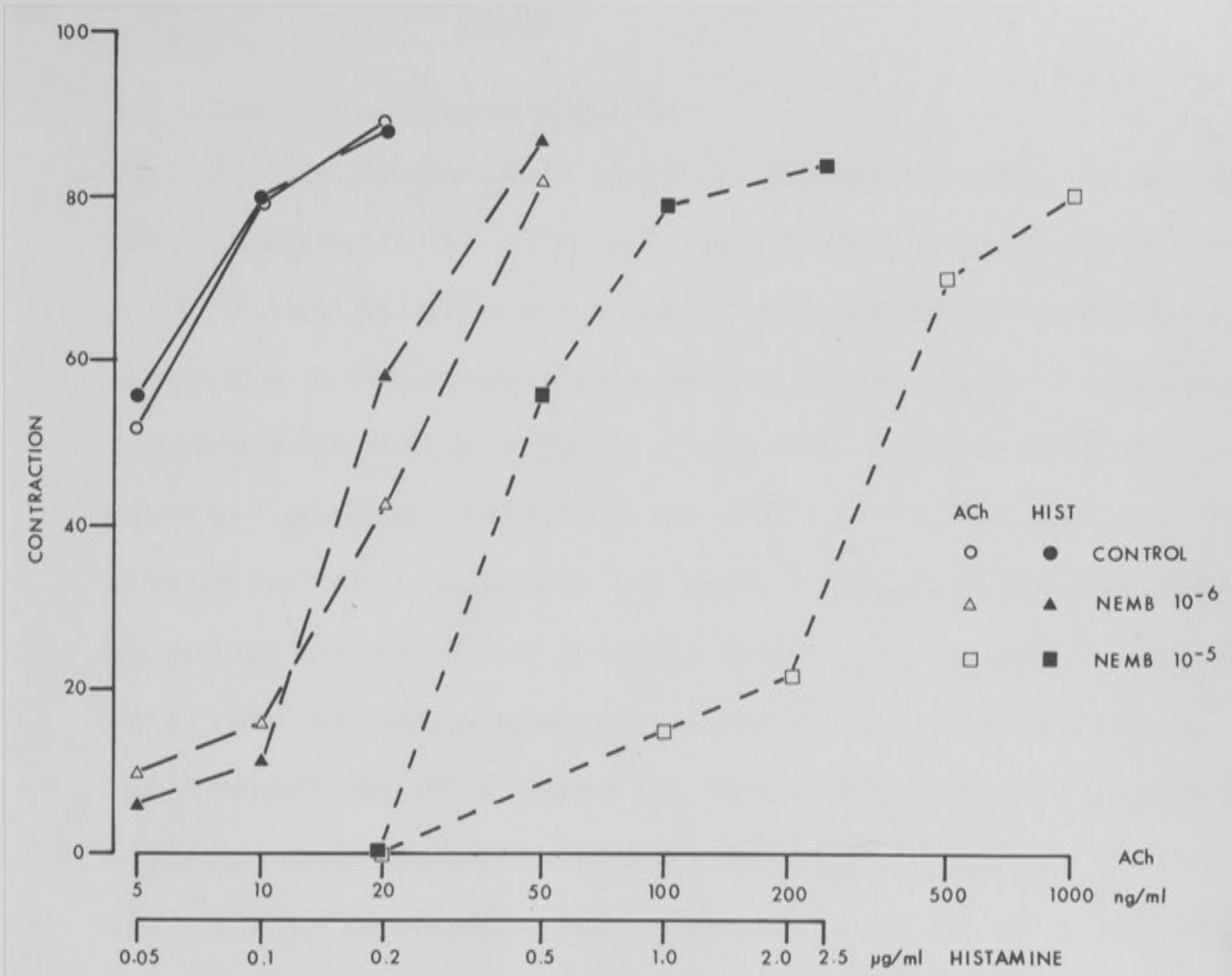

Fig. 54: Contractions (arbitrary scale derived from pen-recorder traces) of a strip of guinea-pig ileum in response to acetylcholine (ACh, 5-1000 µg/ml) and histamine hydrochloride (0.05-2.5 µg/ml, calculated as the base). After the control responses had been taken, the bath solution was changed for one including pentobarbitone (nembutal) in concentrations of 10^{-6} w/v initially, then 10^{-5} w/v.

Ordinate: Contraction of preparation.

Abscissae: Upper - dose of acetylcholine

Lower - dose of histamine.

(g) Summary and discussion

In the preliminary series of experiments, in which cats anaesthetized with pentobarbitone, diallylbarbituric acid or diallylbarbituric acid plus urethane were used, there was a wide variation in the proportion of cholinoceptive Betz cells between different cats. This has been reported also by Krnjević and Phillis (1962, 1963b), who attributed the variation in part to differences in depth of anaesthesia. However, the present experiments indicate that this is not a complete answer, as the systemic administration of a range of anaesthetic agents produced corresponding reduction in sensitivity of pericruciate neurones to DL-homocysteic acid as well as to acetylcholine, and yet there has never been any difficulty in demonstrating amino acid sensitivity of cortical neurones in anaesthetized preparations (Krnjević and Phillis, 1963a; Crawford and Curtis, 1964).

The cholinoceptive neurones in anaesthetized cats show certain consistent features (see also Section V), and this pattern of depth beneath the cortical surface, similar random background discharge, the same order of magnitude of the ACh-ejecting currents required to produce firing, and the same detailed pharmacology of the excitation by cholinomimetics (see also Krnjević and Phillis, 1963c; Krnjević, 1964, 1965a), could be reproduced in certain

cells in non-anaesthetized cerveau isolé preparations (Section V(d) above). This latter population (which was used in almost all the experiments on the action of anaesthetic agents) was assumed to include the Betz cells, although identification by antidromic invasion from the pyramidal tracts above the level of brainstem coagulation was not attempted.

Each of the systemically administered anaesthetic agents (pentobarbitone, thiogenal, diallylbarbituric acid, chloralose, urethane and halothane/air and nitrous oxide/oxygen mixtures) produced comparable effects on both acetylcholine and amino acid sensitivity of pericruciate neurones. Doses ranging from an amount which produced clinical anaesthesia in control animals down to a tenth or a twentieth of this amount were used. Whereas chloralose virtually abolished chemical sensitivity to normal doses of ACh and DLH, with a very slow recovery (not observed in these experiments because of technical difficulty in holding a cell over periods greater than about an hour), small doses of thiogenal, pentobarbitone and diallylbarbituric acid produced reversible diminution of synaptic firing and of DLH- and ACh-responses. The time of maximal effect, and the rate of recovery from each of these agents, corresponded roughly with the clinical anaesthesia produced by each. Urethane and $1\frac{1}{2}$-$2\frac{1}{2}$ per cent

halothane/air mixtures were rather less potent than the barbiturates, and required amounts approximating the full anaesthetic doses to produce appreciable changes in chemical sensitivity of single neurones. Their administration was frequently complicated by changes in blood pressure and consequent slight movement of the cell with respect to the microelectrode tip. Nitrous oxide mixtures (60-90 per cent in oxygen) had little apparent effect on the chemical sensitivity of the cells tested.

The similarity of time-course of anaesthetic depression of the 'background' firing of the cell to that of chemical sensitivity of the postsynaptic membrane (assuming that the ejected drugs indicate this by acting directly on the cell under observation) indicates that the total amount of transmitter released by the activity of presynaptic cells is depressed with the same time course as is the chemical sensitivity of the observed cell, or else that the type of cell under observation was so heavily depressed by the anaesthetic that synaptic excitation from other, less affected, cells failed to fire it until the time both electrophoretically-ejected agents were also able to do so.

The testing method used (alternate ejection of two excitants, with extracellular recording of the spikes of

one major unit, which was assumed to be directly excited by each agent) did not permit evaluation of any presynaptic effect of the anaesthetics. These may well be important additional mechanisms for any given agent, superimposed on the postsynaptic cell depression common to all anaesthetics. For instance, Mitchell (1963) demonstrated interference by chloralose with the release of acetylcholine from the cortex, and Løyning, Oshima and Yokota (1963) report that the excitatory postsynaptic potential of spinal motoneurones is reduced by thiamylal. However, they have not excluded possible postsynaptic effects of the anaesthetic upon the conductance change due to the transmitter (cf. Maeno and Edwards, 1965).

Because of considerable variation in the chemical structure of the anaesthetics used, it seems unlikely that they could all be interfering competitively to similar degrees with the combination of acetylcholine, the amino acid, and the synaptic transmitter with their appropriate receptors. Rather it would seem that the anaesthetics act at some point on the pathway between membrane depolarization (whether an EPSP or that due to a chemical excitant unrelated to synaptic transmission) and the generation of the spike. This possibility was earlier suggested by Shanes et al. (1959) for the action of local anaesthetics upon the axon membrane, and is the third

alternative postsynaptic action of anaesthetics proposed in the introduction to this Section. However, a specific interference with, say, sodium conductance changes in the initial depolarization by the excitants (Maeno and Edwards, 1965) would again affect all forms of excitation of the cell, and such a mechanism cannot be excluded as a basis for anaesthetic action.

Recent studies of the effect of various general anaesthetic agents on the excitability of spinal motoneurones and interneurones (Shapovalov, 1964) indicate that these agents act upon the postsynaptic cell membrane to reduce its excitability to direct and synaptic stimulation, and this finding is compatible with much indirect evidence from motoneurones (Somjen and Gill, 1963), monosynaptic spinal reflexes (Somjen, 1963) and isolated frog spinal ganglia (Aceves and Machne, 1963). This effect would again be compatible with an uncoupling of the Na^+ conductance changes involved in spike generation from the initial depolarization which normally triggers them. Intracellular recording of the membrane depolarization produced by electrophoretic ejection of the amino acids and ACh in the presence of anaesthetic is a necessary further step in the investigation.

Perhaps the most striking feature of these experiments was the difference between the systemic (i.v.) and

electrophoretic administration of pentobarbitone on the excitation by DLH and ACh. As the distribution of systemically administered agents is approximately uniform over the whole cell surface and the responses to both excitants are then similarly affected, the greater susceptibility of DLH than ACh responses when tested by local electrophoretic ejection of barbiturate must reflect the higher relative concentration of anaesthetic to which the amino acid receptor membrane is thereby exposed. Considerably larger concentrations or more prolonged administration of pentobarbitone will reduce the response to acetylcholine also, suggesting that diffusion of the anaesthetic eventually occurs to the ACh-receptors and their surrounding membrane. If, in order to record reasonable-sized extracellular spikes, the microelectrode tip has to be fairly close to the cell soma or large proximal dendrites, it follows that at least some of the ACh-receptors are more remote, e.g., on the dendrites. The relatively large doses of barbiturate or procaine needed to reduce acetylcholine-induced excitation also frequently caused changes in the extracellular spike, presumably by rendering inactive portions of the cell membrane close to the electrode. These results are complementary to those with local and systemic atropine,

and similar conclusions as to the probable dendritic location of ACh-receptors were reached in the discussion of Section VIII(f).

A depressant effect of electrophoretically ejected barbiturates on single cortical neurones was also reported by Krnjević (1965a) who found both diallylbarbituric acid and pentobarbitone to be rather potent, with a fairly slow action, from which the cells recovered in 15-30 seconds. Chloralose was a rather less active depressant in his series than when given systemically in the present experiments.

Spike changes were common with large doses of pentobarbitone and with smaller amounts of phenobarbitone. As they were also produced by ejection of procaine, they were attributed to a local anaesthetic-like action. With phenobarbitone, it was difficult to separate the effects on chemical sensitivity from those on spike generation, and this fact probably accounts for the relative ease with which the acetylcholine-excitation was reduced. Large doses of systemically administered anaesthetics also produced spike alteration, in many cases no doubt by effects on blood pressure, but possibly in part by direct effects upon the cell membrane. This possibility also requires further investigation.

Unfortunately, the intravenous administration of the local anaesthetics procaine and procaine amide failed to produce a 'non-specific' reduction in chemical sensitivity of cortical neurones, and in their synaptic background excitation. This may be due to a failure of these compounds to reach the cell studied in a sufficiently high concentration. When given intravenously, procaine amide also failed to affect the chemical sensitivity of cerebellar neurones.

In conclusion, despite reports of particular pharmacological effects of various anaesthetics on postsynaptic cell membranes of spinal neurones (see Marley and Vane, 1963; Haase and van der Meulen, 1963; Biscoe and Krnjević, 1963) it has not been possible to demonstrate similar effects on the excitation of cortical neurones by ACh and DLH. All the general anaesthetics tested produced a similar 'non-specific' reduction in chemical sensitivity of pericruciate neurones, apparently by a mechanism involving the conductance changes common to each excitant, or those involved in spike generation and propagation in the postsynaptic cell membrane.

SECTION XI - GENERAL DISCUSSION

In any attempt to equate the actions reported in earlier Sections for the amino acids or acetylcholine with synaptic transmitter roles at cortical neurones, it is necessary to demonstrate the natural occurrence of the supposed transmitter substance in the central nervous system, together with enzyme systems for its production (criterion (a) of Section I above).

(a) Amino acids as synaptic transmitters

The natural occurrence of L-glutamic and γ-amino-butyric acid, and their important role in the metabolism of neurones, appears undoubted (e.g. Roberts and Frankel, 1950; Tallan, Moore and Stein, 1954; Waelsch, 1957, 1962; Roberts, 1961, 1962). Furthermore, it has recently been shown by Jasper, Khan and Elliott (1965) that both these amino acids are released from the cerebral cortex in amounts which are related to the state of activity of cortical neurones, as shown by the electrocorticogram. However, this finding does not establish that the release of the amino acids is from synaptic endings within the cortex (cf. criterion (c) of the Introduction of this thesis).

Recently Krnjević (1964, 1965a) has concluded that 'there is no really strong evidence against the possibility that L-glutamate may be the principal excitatory transmitter in the cerebral cortex and perhaps in the whole central nervous system' (Krnjević, 1965a, p.10) and that the properties of GABA are 'very much what would be needed for an inhibitory transmitter' (op.cit., p.11), although his experiments with Phillis (cited in Krnjević, 1964) indicate that GABA did not appreciably alter the membrane potential of Betz cells. In favour of the proposed transmitter roles, Krnjević adduces the strong and rapidly reversible actions of the two amino acids, their presence in the brain in large amounts and their ability to affect all cortical neurones, as well as the fact that L-glutamate and GABA appear to be involved in neuromuscular and inhibitory transmission respectively in the Crustacea (Takeuchi and Takeuchi, 1964; Boistel and Fatt, 1958; Kravitz, Kuffler and Potter, 1963). It is of interest also that the actions of these amino acids can be detected from the earliest stage in development at which unit responses can be evoked in cerebral cortex (in the case of the kitten, from the first week after birth) (Krnjević, Randić and Straughan, 1965; cited by Krnjević, 1965a).

On the other hand, at least in the case of spinal motoneurones, GABA and L-glutamate produce different changes in membrane conductance from those of synaptic

activation (Curtis, Phillis and Watkins, 1959; Curtis, 1962a, 1965). As mentioned above Krnjević also reports that extracellular ejection of GABA fails to hyperpolarize Betz cells, although hyperpolarizing IPSP's are well known to occur with these cells (e.g. Phillips, 1956, 1959, 1961; Lux and Klee, 1962; Klee and Lux, 1962; Purpura and Shofer, 1964; Purpura, Shofer and Musgrave, 1964). Thus, where intracellular comparisons have been made to date, it would not appear that GABA and L-glutamate are acting as synaptic transmitters. Then, by analogy, their virtually identical actions upon other central neurones (Sections III and IV) cannot be ascribed to a transmitter action at these latter sites either. However, Eccles (1964, p.72) remarks that the excitatory transmitter substance may be related to the acidic amino acids, the discrepancies in the equilibrium potentials arising because the permeability change produced by L-glutamate is biased slightly more for K^+ relative to Na^+ than that produced by the transmitter. There is no positive evidence as to the correctness of this hypothesis, and all that can be said as yet is that the transmitter for monosynaptic excitation of spinal motoneurones appears to be an anion, on the basis of changes produced in the EPSP by the passage of hyperpolarizing currents across the cell membrane (Curtis and Eccles, 1959; Eccles, 1964, pp.73-4).

As was noted in Section IV(d), the neutral amino acids produce a conductance change in the Betz cell membrane, and are not merely blocking the excitatory synapses (cf. Purpura et al., 1957, 1959; Purpura, 1960). In the comparison of potencies of these depressant amino acids, however, they were tested against the excitation produced by DL-homocysteic acid. Under these conditions, not only will both DLH and the depressant presumably be producing their conductance changes in the same area of membrane, but competition of the two amino acids for the same receptor may also be involved in the observed reduction of the DLH-firing rate. Weak depressants such as glycocyamine are able to reduce amino acid evoked firing of spinal interneurones without affecting their synaptic excitation (Section IV(b)v; see also Curtis and Watkins, 1960; Krnjević and Phillis, 1963a). This again may be an indication that the amino acid receptors are distinct from those involved in synaptic transmission, although the synaptic excitation may simply have been too powerful to have been overcome by the small conductance change due to this depressant.

To date, histochemical techniques have not contributed much to the evaluation of a possible synaptic role of the amino acids. However, even the potential usefulness of histochemistry may be limited by the high intracellular

concentration of the active amino acids (Tallan, Moore and Stein, 1954; Waelsch, 1962). For instance, it has been estimated (Curtis and Watkins, 1965) that only 0.1-1.0 per cent of the total intracellular glutamate need be relocated outside the cell in order to affect its activity, assuming an extracellular concentration of 10^{-4} to 10^{-3} M to be effective in causing excitation (see also Curtis, Phillis and Watkins, 1960; Krnjević and Phillis, 1963a; Krnjević, 1965a). However, the recent development of a suitable technique (van Gelder, 1965a) has led to the demonstration of enzymes metabolizing GABA in many neurones of the grey matter of the cerebrum, cerebellum and spinal cord, as well as in cells in contact with the cerebrospinal fluid and the bloodstream (van Gelder, 1965b). Thus, the method has already led to a direct demonostration of a functional blood-brain barrier system for this amino acid, and is of considerable interest for future development. Furthermore, the presence of a high GABA-content in motoneurones and other non-inhibitory cells casts doubt on the proposed inhibitory transmitter role for this amino acid.

(b) Acetylcholine as a cortical synaptic transmitter

Acetylcholine and related compounds produce a fairly slow excitation of certain susceptible neurones of the

cerebral, cerebellar and hippocampal cortices, and this excitation is frequently preceded by a phase of decreased excitability (Sections V and VI). In general, muscarinic substances are more potent than nicotinic (Section VI), and these effects are blocked by atropine but not by curariform agents (Sections VII and VIII). An exception to this latter statement must be made in the case of certain Purkinje cells whose ACh-sensitivity is reduced by DHβE.

The exact receptor sites involved in these reactions are unknown. It does not seem likely, however, that the effects of the cholinomimetics can be due to an indirect action, e.g. upon blood vessels (cf. Schlag, 1956), as both ACh-sensitive and nonresponsive neurones were found in close proximity to each other in virtually all tracks. Neither would it appear that the cholinoceptive areas can form a major part of the constructional membrane of neurones, as it would then be expected that all neurones would respond to these excitants, in a similar manner to the 'non-specific' actions of the amino acids (Curtis and Watkins, 1963). Only the 'large efferent cell' populations (Betz, Purkinke and hippocampal pyramidal neurones) show a high proportion of cholinoceptive units, however, and even amongst these populations it is rare to find that all cells tested in a given cat are fired

by ACh. This variation in the percentage of cells fired by cholinomimetics is not simply attributed to variation in anaesthetic state, as in two unanaesthetized cerveau isolé cats it proved impossible to find any ACh-sensitive pericruciate neurones (one experiment) or Purkinje cells (one experiment). Both of these cats had normal cortical circulations and a normal systemic blood pressure, and the neurones responded normally to electrophoretically-ejected amino acids throughout the experiments. Several different microelectrodes containing ACh were tried during these experiments, and it is unlikely that in each 'capping' of the electrode by cellular debris (Andersen and Curtis, 1964a) could preferentially block the ejection of ACh.

In contrast to these experiments, however, in several cats every Betz or Purkinje cell tested was found to be cholinoceptive. Possibly all large cortical efferent cells do possess cholinoceptive sites distributed over their surface area, including regions relatively distant from the usual position of the electrode tip near the cell body. (Circumstantial evidence is given in Sections VIII(f) and X(g) for this proposed location of the ACh-receptors, and a similar disposition is suggested for thalamic receptor sites by Andersen and Curtis, 1964a). The variation from 0 to 100 per cent observed in the

proportion of cholinoceptive cells to the total of units tested might then be due to the process of 'sampling' the 10-20 identified cells examined in any one experiment. Diffusion of the cholinomimetic to its receptors may have been prevented by the geometrical arrangement of glia around the neurone, or the microelectrode may accidentally have been placed so far from the ACh-sensitive areas that an adequate drug concentration was not reached at these areas.

An explanation of the apparent insensitivity of some Betz or Purkinje cells to ACh in terms of geometric factors appears preferable to a hypothesis that ACh-sensitivity reflects the impingement of an afferent cholinergic pathway upon some of these neurones but not on others. If the response to ACh were related to synaptic function, it is difficult to see why all cells of the appropriate type would not manifest it, particularly in the case of Purkinje cells where the afferent excitatory systems (parallel and climbing fibre synapses) are the same for every cell. Even if the ACh-receptors are extra-synaptic it is hard to understand why all cells of a given type in the same cat, being histologically and functionally the same, should not all possess the appropriate receptor areas. Genetic or constitutional factors in a given cat might cause the disappearance of

the ACh-sensitivity of the cells, but would be unlikely to produce intermediate proportions of cells with functional ACh-receptors.

The slow onset of excitation by ACh and the cholinomimetics is due in part to the time taken for diffusion to the receptor areas. In the cerebellum both DLH and carbachol were found to diffuse at rates of about 10μ/second (Section VI(c)) and, if acetylcholine behaves similarly, an effective concentration 'front' of ACh could traverse a distance of some 50-300μ from the electrode tip in the observed latent periods. Not all of the observed delay is due to diffusion time, however. The muscarninic component of ACh-action on the Renshaw cell (Curtis and Ryall, 1964, 1965a-c) has a latent period of several seconds although diffusion distances in this case cannot be extremely large. Furthermore, the preliminary depression of cortical neurones by ACh will delay the onset of ACh-excitation to some extent. On the other hand, the short latency of onset of firing observed with amino acids such as L- and D-glutamate implies that their receptors must be close to the electrode tip (see also Andersen and Curtis, 1964a).

In conclusion then, whether the ACh-sensitive areas are or are not subsynaptic, at least some of them appear to be further from the recording site than are the amino

acid receptors, and the variation in prevalence of cells which respond to cholinomimetics appears due to accidental geometric factors limiting the diffusion of the drug to these areas. The possible synaptic role of ACh will now be examined separately for the cerebral and cerebellar cortices in the light of certain reported histochemical evidence, and the studies made with ACh-antagonists (Sections VII and VIII).

(i) Cerebral cortex. Acetylcholine is known to be present within the cerebral cortex, and to be released from it in amounts which correlate with the state of cortical activity (MacIntosh, 1941; Elliott, Swank and Henderson, 1950; MacIntosh and Oborin, 1953; Mitchell, 1963; Szerb, 1963). The subcellular distribution pattern indicates that the ACh is associated with synaptic vesicles in the nerve-ending fractions of brain homogenates (Hebb and Whittaker, 1958; de Robertis et al., 1962, 1963; Whittaker, Michaelson and Kirkland, 1963, 1964; Ryall, 1964). Choline acetylase also is concentrated in the nerve endings, possibly in association with the vesicles themselves (de Robertis et al., 1963) or merely in the cytoplasm of the endings (Whittaker et al., 1963, 1964). In either event, the location of the enzyme system which synthesizes ACh would be suitable for possibly cholinergic transmission.

Quite large amounts of cholinesterases can be shown to be present in the cytoplasm of some central neurones (e.g. Snell, 1961; Koelle, 1963). Histochemical techniques have also shown AChE to be associated with the neuronal membrane in various central neurones (for a review of these findings, see Koelle, 1963), but it has not yet proved possible to localize selectively the external and internal AChE of central neurones in the manner demonstrated for autonomic ganglia by Koelle and Koelle (1959). The results of subcellular distribution studies of this enzyme (de Robertis et al., 1962, 1963) are also compatible with localization of AChE upon the synaptic membranes, and thus with a transmitter role for ACh.

By analogy with cholinergic neurones of the peripheral nervous system (see, for example, Hebb, 1957, 1961) many workers use the shorthand descriptive term 'cholinergic' for central neurones and tracts which contain acetylcholinesterase (e.g. Snell, 1961; Shute and Lewis, 1961, 1963, 1964; Krnjević and Silver, 1963b; Krnjević, 1965b), although it is well realized that acetylcholine rather than cholinesterase is the identifying marker of cholinergic transmission (Dale, 1937; Feldberg, 1957; Hebb, 1957, 1961). Karczmar (1963) has extensively reviewed the developmental significance of cholinesterases, and their relation to neurogenesis and function in various species.

In any attempt to evaluate a transmitter function for ACh, studies with both the synthesizing and destructive enzymes of the ACh-system must be correlated, and in the following discussion quotation marks will be placed around the term 'cholinergic' when used in reference to pathways determined on histochemical (AChE) evidence.

The majority of neocortical neurones fail to stain for AChE (Koelle, 1963; Krnjević and Silver, 1963a) but a faint to moderate staining of many deep pyramidal cells in layer V has been reported (Krnjević and Silver, 1963a). Undercutting of the cortex results in a substantial decrease in the intensity of this staining, and a concurrent loss of acetyltransferase activity of the cortex (Hebb, Krnjević and Silver, 1963). These results were taken to indicate a 'cholinergic' innervation of the pyramidal cells of the pericruciate and suprasylvian cortex from distant cell bodies, and the staining of the perikarya of the pyramidal cells was attributed to the fine terminal meshwork of this system (Krnjević and Silver, 1963a; Krnjević, 1965b).

Possible sources of this 'cholinergic' system are the arcuate fibres around the bottom of sulci which link each gyrus with its neighbours (Krnjević and Silver, 1963b), the scattered AChE-staining fibres of the internal capsule (Krnjević and Silver, 1963a) or the forebrain component

of the reticular formation (Shute and Lewis, 1963a, b). On physiological evidence, Krnjević and Phillis (1963b) suggest that the cholinergic pathway may mediate the late repetitive discharge which follows sensory volleys (Adrian, 1941; Morison and Dempsey, 1943; Bremer and Bonnet, 1950). Krnjević and Phillis (op.cit.) did not find any specific association between the cholinoceptive cells and excitation by direct afferent activity from thalamic relay cells, by stimulation of midline thalamic nuclei, or by transcallosal volleys, although many of the cells could be activated by these routes. Szerb (1965) has also produced evidence in favour of a cholinergic component of the sensory evoked potential by studying the effects of atropine and hemicholinium on the averaged cortical potentials.

In the present experiments no attempt was made to define the possible cholinergic pathways, although the failure of systemic or local administration of atropine to affect the synaptic 'background' of cholinoceptive units suggests that the cholinergic contribution to this excitation was small under the (non-stimulated) conditions employed. On the other hand, Krnjević and Phillis (1963c) have found substantial reduction in spontaneous activity of cholinoceptive neurones after doses of some 0.2 mg./kg. atropine.

An alternative explanation of the negative findings with atropine on the background firing, although the sensitivity of the cell to ejected ACh was virtually abolished, would be that the ACh-receptors are extra-synaptic and play no part in the regulation of normal activity of the Betz cells. This hypothesis would mean that histochemical demonstration of AChE must be disregarded as irrelevant to the demonstration of a synaptic transmitter role for ACh. On the other hand, it is well recognized that at many synapses the responses to added ACh are more readily blocked by antagonists than are the effects of nerve stimulation (e.g. Dale, 1938; Ambache, 1955), so such a drastic relegation of the histochemical evidence may be unnecessary.

To summarize, it is impossible to state with certainty that the sensitivity of Betz cells to cholinomimetics is indicative of a cholinergic pathway impinging upon them. If such a pathway exists, then it probably contributes little direct excitatory barrage to the cells in the absence of specific sensory stimulation. Possibly the forebrain component of the reticular system (see Shute and Lewis, 1963) contributes a cholinergic input to the background dendritic depolarization of Betz cells, resembling the 'non-specific e.p.s.p's' of these cells from midline thalamic nuclei (Creutzfeldt and Lux, 1964).

Upon spinal motoneurones also, the reticular system appears to act on the dendrites, although in this case the reticular influence is inhibitory rather than excitatory (see Llinas and Terzuolo, 1965).

(ii) Cerebellum. The general remarks made above, on the need to correlate histochemical evidence of AChE-distribution with the demonstration of the synthesis of acetylcholine before conclusions are drawn as to its possible synaptic role, apply with particular force in the cerebellar cortex.

Numerous workers have investigated the choline acetylase activity and ACh concentration of the cerebellum (e.g. MacIntosh, 1941; Hebb and Silver, 1956). In general, these values are surprisingly low in relation to the cholinesterase activity of this region, although choline-acetylase activity in the middle and inferior peduncles would be consistent with at least some afferent cholinergic tracts (see Hebb, 1961). Recent histochemical determination of AChE-activity in the cerebellar peduncles (Shute and Lewis, 1965; Phillis, 1965a, b) also supports the contention that both afferent and efferent pathways from the cerebellum include some 'cholinergic' fibres. Marked species differences exist in the disposition of AChE between the layers of the cerebellar cortex (Friede and

Fleming, 1964), making comparisons between species particularly hazardous as a means of elucidating the function of ACh.

Afferent fibres to the cerebellum are of two types - mossy fibres, whose characteristic endings form the cerebellar 'glomeruli' or 'rosettes' in the granular layer with the claw-like dendrites of granule cells and the telodendria of the Golgi type II cells, and climbing fibres whose main termination is an intertwining with the smooth main dendrites of Purkinje cells (see Scheibel and Scheibel, 1954; also Fox, 1962). The climbing fibres originate from the inferior olive (Szentagothai and Rajkovits, 1959) and are distributed on a one-to-one basis with the P-cells, whereas all other afferent projections to the cerebellum end as mossy fibres. The synaptic organization is of importance as, if mossy fibres be cholinergic the granule cells with which they form synapses must be cholinoceptive, whereas if the cholinergic afferent pathways ended in climbing fibres only Purkinje cells need be responsive to ACh.

The first of these alternatives has been favoured by Phillis and his colleagues (Austin, Phillis and Steele, 1964; McCance and Phillis, 1964a, b; Phillis, 1965a). These workers report the presence of cholinoceptive units in the granular layer of the cat cerebellar cortex, and

have been unable to find a comparable proportion of identified P-cells which were responsive to ACh (McCance and Phillis, 1964b). However, there exists some doubt as to the identification of the cell types involved in these experiments (see Section V(g)). Furthermore, a corollary of the hypothesis that mossy fibres are cholinergic would be that the parallel-fibre system (granule cell axons) could be excited by ejection of cholinomimetics into the granule cell layer. Experiments using two microelectrodes (Section VI(c)) were devised in an attempt to demonstrate such indirect activation of Purkinje and basket cells, but failed to show any effect of ACh or carbachol on granule cells although DL-homocysteic acid was effective in firing them. The failure of atropine and dihydro-β-erythroidine to modify the potentials evoked in the cerebellum by lateral reticular, external cuneate and pontine nuclear stimulation also casts some doubt on the role of ACh in transmission at the mossy-fibre terminations of these pathways.

Recently, Israel and Whittaker (1965) have determined the ACh-content of synaptosomes from cerebellar homogenates of various species. Large (2-5μ diameter) synaptosomes are presumably derived from mossy fibre endings, while smaller (0.5μ diameter) endings appear to represent parallel-fibre synapses. In the guinea-pig

and rat the larger synaptosomes contain more ACh than the smaller, but in the cat and pigeon this distribution is reversed. These findings are in accordance with the histochemical (AChE) evidence for the guinea-pig, rat and pigeon (cf. Friede and Fleming, 1964), but for the cat there is less ACh in the mossy fibre endings than expected from the AChE-staining. Thus, neither the distribution of acetylcholine nor the pharmacological findings of the present investigation (Sections V-VIII, and discussion above) support the suggestion of cholinergic function of mossy fibres in the cat.

Phillis' experiments (1965b) with pedunculotomized cats, and cats in which the vermal cortex was undercut, failed to show any decrease in the AChE-staining of the cells and synaptic areas of the granular layer. These findings may reflect intracortical 'cholinergic' circuits in the cat cerebellum which are not present in the rat (cf. Mead and van der Loos, 1964).

Leaving for the moment the other possible type of 'cholinergic' afferent pathway, the climbing fibres, it is of importance to consider whether Purkinje cells are cholinoceptive because of impingement of cholinergic parallel-fibre synapses. The results of Israel and Whittaker (1965) would be compatible with this suggestion for the cat, but the histochemical findings are not.

Friede and Fleming (1964) report little or no reaction for AChE in the molecular layer of the cat, and although Phillis (1965b) finds considerably more AChE in this layer using a 'direct-colouring' method (Karnovsky and Roots, 1964) than is demonstrable by the Gerebtzoff modification of the thiocholine technique (see Gerebtzoff, 1959), there seems to be no association between this AChE activity and neuronal structures in the molecular layer (Phillis, 1965b, p.268). Furthermore, the parallel fibres end not only on Purkinje cell dendrites, but also upon basket cell and Golgi cell dendritic trees. On the suggestion by Dale (1935) that the same transmitter is released at all terminals of a given neurone ('Dale's Principle'; Eccles, 1957, 1964), one would expect that both basket and Purkinje cells would be cholinoceptive if the parallel fibres were cholinergic, but the experiment of Fig. 26 proves that this is not the case (Section VI(d)). Therefore the parallel fibre system is not cholinergic, and the sensitivity of P-cells to ACh is unrelated to these synapses. Further evidence on this point is provided by the failure of atropine and DHβE to block the responses to local parallel fibre stimulation.

One major synaptic pathway remains to be discussed - the climbing fibres (Scheibel and Scheibel, 1954; Szentagothai and Rajkovits, 1959; Fox, 1962). These fibres

arise in the inferior olive, in the cells of which Holmes and Wolstencroft (1964) have described the presence of AChE in the cat. No such concentration of this enzyme exists in these nuclei in the rat (Koelle, 1954, 1963), although Gerebtzoff (1959, p.87), using a technique which has been criticized on various grounds (see Karczmar, 1963, p.151), reports light AChE-staining of this nucleus. However, as indicated above histochemical evidence is not direct proof of a synaptic function of ACh, and as yet no confirmatory studies have been made of the cholineacetylase activity of the inferior olive. Again atropine failed to modify the responses in the cerebellar vermis to climbing-fibre stimulation (Fig. 30), although the activation of P-cells by this system is so intense (Eccles, Llinas and Sasaki, 1964, 1965a, b) that the antagonist may fail to block it even if transmission were by means of acetylcholine.

In summary, then, neither mossy nor parallel fibres in the cat cerebellum appear to be cholinergic. There is insufficient evidence to associate the sensitivity of Purkinje cells towards acetylcholine with a cholinergic function of the climbing fibres, although this remains a possibility. It is also conceivable that the ACh-receptors of the P-cells are extrasynaptic, as was suggested above in the case of Betz cells.

(c) Comments on the actions of anaesthetic agents

All the non-volatile general anaesthetics tested in these experiments showed a common mode of action, i.e. a depression of postsynaptic excitability towards chemical agents (including the synaptic transmitter). The two volatile anaesthetics, nitrous oxide and halothane, had considerably less effect on the chemical sensitivity of Betz cells, and may be acting at other sites (perhaps even presynaptically). This difference between the volatile and non-volatile agents may reflect a different mode of action, as was found with various agents on nerve conduction (see Lorente de Nó, 1947; Schoepfle, 1957; Shanes, 1958), or may merely indicate that Betz cells are somewhat more resistant to the volatile anaesthetics than are the cells first affected during the production of the anaesthetic state. Nitrous oxide and halothane were tested only in concentrations which produced clinical anaesthesia (Section X(c)), and it is possible that slightly higher concentrations would have produced the same effects on Betz cells as did the barbiturates, urethane and chloralose. Previous studies on the production of the clinical state of anaesthesia have shown initial effects of the anaesthetic agents upon the reticular formation (e.g. King, 1956; Brazier, 1961; Clutton-Brock, 1961; Killam, 1962). Differences in effect between various

anaesthetic agents have also been observed in these experiments (King, 1956; Domino and Ueki, 1959; Brazier, 1961). However, in the present experiments, none of the agents employed showed specific antagonism towards either chemical excitant (cf. also Marley and Vane, 1963, where pentobarbitone was found to interfere with the effects of tryptamine on spinal reflexes although other anaesthetics did not). Such additional 'specific' effects of anaesthetic agents may be manifest towards a particular excitant under appropriate circumstances, but will as a rule be secondary to the depression common to all anaesthetics.

A great deal of further investigation is needed on this subject, including the use of intracellular recording techniques in an attempt to define the membrane properties of central neurones affected by general anaesthetics.

(d) Concluding remarks

The aim of this thesis has been to confirm and extend pharmacological studies of single cortical neurones, and to correlate the findings with other available evidence in an attempt to define possible synaptic transmitter substances acting at these sites.

Neither L-glutamic acid nor γ-aminobutyric acid can satisfactorily be shown to be central transmitters, and although acetylcholine may have a synaptic function upon

the large cortical efferent cells, there is no overwhelming evidence as to the pathways involved. In the cerebellum, neither mossy fibres nor parallel fibres appear to be cholinergic, despite some indirect histochemical evidence. In fact, it now appears that neither the presence of destructive enzymes nor the excitation of a cell by a supposed transmitter can be taken as sufficient indication of a synaptic role. All the criteria outlined in Section I are necessary for such an identification.

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TABLE I - Excitant Amino Acids on Cortical Neurones

Amino acid	Potency			Latency (secs)	
	Mean	Range	Tests	Onset	Offset
N-methyl-D-aspartic	6.5	2-16	24	15-30	2-10
DL-Homocysteic	4	2.5-6	23	10-20	2-5
N-Propyl-D-aspartic	1.2	1-1.5	6	10-15	10-20
L-Glutamic	(1.0, Standard)			0.5-1.0	0.5-1.0
L-Aspartic	1	1-1.2	5	"	"
D-Glutamic	0.7	0.5-0.8	16	"	"
D-Aspartic	0.8	0.7-1.0	5	"	"

For further description of potency estimation, see text (Section III (b)). Latencies were measured from filmed records except for values of 5 seconds or more, which were readily estimated from the 'Recti/Riter' pen-recorder traces.

TABLE II - Intraventricular Injection of Excitant Amino Acids

Amino Acids	No. of mice	Dose (μg/0.05 ml.)	Effect
N-Methyl-D-aspartic acid	4	10	L
	9	2-5	+ + +
N-Methyl-DL-aspartic acid	4	20-50	L
	6	5-10	+ + +
	5	2	+ +
N-Methyl-L-aspartic	4	50	+ +
	2	20	+
D-Homocysteic acid	8	20-50	+ + +
	2	10	+ +
DL-Homocysteic acid	8	25-50	+ + +
	4	10	+ (+)
L-Homocysteic acid	6	20-50	+
N-Methyl-DL-glutamic acid	4	50	+ + (+)
	2	20	+
D-Aspartic acid	5	50	+
L-Aspartic acid	5	50	+
L-Glutamic acid	2	150	+
	6	50-100	Nil

L: Lethal excitant effects - clonic flexor convulsions, followed by extensor spasm and death.
+++: Gross hyperactivity, occasional convulsions.
++: Mild to moderate hyperactivity.
+: Slight excitant action. For further description see text.

TABLE III - Neutral Amino Acids and Related Compounds

Amino acid	Concn. (M)	pH	Potency
3-Aminopropane sulphonic acid	0.2	9-10.6	- - - -
γ-Aminobutyric acid	1.2	2.9-7.2	- - -
γ-Amino -β-hydroxybutyric acid	2	3.1	- -
Taurine	0.8	8.5-10	- (-)
γ-Hydroxybutyric acid	1	7	0
β-Guanidinopropionic acid	1	3	- -
Guanidinoacetic acid	0.025	3-3.1	-
ε-Aminocaproic acid	0.5	3.1, 6.8	- *
ω-Aminocaprylic acid	0.5-1	3.0, 7.7	0 *
L-Asparagine	Sat.	3.1-4.5	0 *
D-Asparagine	Sat.	3-5	0

Potency is relative to GABA (- - -).

* Late excitation with solutions of low pH (see text — Section IV (b) vi and IV (c) i).

TABLE IV - Intraventricular Injection of Depressant Amino Acids

Amino Acids	No. of mice	Dose (μg/0.05 ml.)	Effect
3-Amino-1-propane-sulphonic acid	8	100	- - -
	4	50	- -
	4	20	-
γ-Amino-n-butyric acid	6	150-250	- - -
	6	100	- -
	4	50	Nil
Taurine	13	100-150	- -
	6	20-50	Nil

- - - : Gross incoordination and loss of tone, with tendency to fall into sleep-like state.
- - : Incoordination, loss of muscle tone.
- : Minimal depressant effects on behaviour.

TABLE V - Acetylcholine-Sensitivity of Pericruciate Neurones

Spontaneous activity	ACh-excitation	Betz	'Possibly' Betz	Non-Betz	Unidentified	Total
+	+	57	10	10	1	78
+	-	28	3	5	-	36
-	+	14	2	1	2	19
-	-	40	3	49	6	98
Total:		139	18	65	9	231

The criteria for identification of the Betz cells were as outlined in Section II (h) i. Those cells listed above as 'unidentified' did not have their responses to pyramidal tract volleys tested.

Of 114 cells of all types showing moderate to high rates of random spontaneous activity, 78 (68.4 per cent) were excited by acetylcholine. 117 cells showed activity <5 spikes per second, or barbiturate 'spindles'. Of these, only 19 (16.2 per cent) were fired by ACh.

TABLE VI - Acetylcholine-Sensitivity of Neurones in Cerveau Isolé Cats

	Cells excited by ACh or carbaminocholine	Non-cholinoceptive cells
Cells possessing random spontaneous activity	38	7
No spontaneous activity	2	42

38 out of 45 spontaneously active cells (84.4 per cent) are cholinoceptive, but only 2 of 44 quiescent cells ($\simeq$ 4.5 per cent).

TABLE VII - Summary of Cholinomimetic Actions on Cortical Neurones

Drug	Pericruciate Cortex		Cerebellar Cortex			Hippocampus
	Betz	Other	Purkinje	Other	Granule	
ACH	+ + + 71/139	+ + + 26/92	+ + + 40/53	+ + + 20/63	0	+ + + 11/19
Carbamino-choline	+ + + (+) 17/20	+ + + 28/51	+ + + + 60/68	+ + + + 39/56	0	+ + + + 17/26
Acβ MeCh	+ + + (+) 6/6	+ + + (+) 9/9	+ + + 3/5	+ + + 2/4	0	+ + + 2/2
PrCh	+ 2/3	N.T. 0/1	N.T.	N.T.	N.T.	N.T.
BuCh	0/3	N.T. 0/1	N.T.	N.T.	N.T.	N.T.
Nicotine	+ + 3/5	+ + 2/3	+ + (+) 4/7	+ + (+) 3/5	N.T.	+ + 2/5
dl-Musca-rine	+ + + + 4/4	+ + + + 3/3	+ + + + 3/3	N.T.	N.T.	N.T.

The potencies are estimated relative to that of ACh, ranked as + + + on each type of neurone. Beneath each potency, the figures give number of cells excited (numerator) in total number of that type tested (denominator).

0: No excitant action
N.T.: Not tested on that type of neurone
AcβMeCh: Acetyl-β-methyl choline
PrCh: Propionylcholine
BuCh: N-Butyrylcholine

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